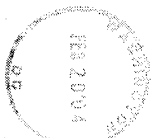


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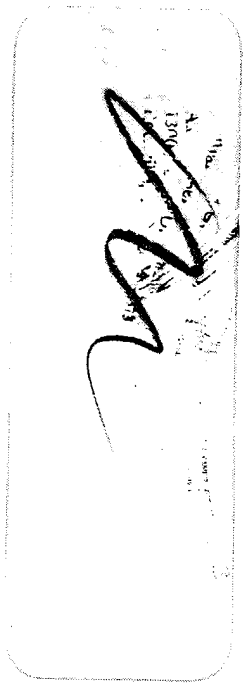
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Martin G. Sirois, et al.
Serial No.: 09/945,131
Filed: August 31, 2001
For: LOCALIZED OLIGONUCLEOTIDE THERAPY FOR
PREVENTING RESTENOSIS
Group Art Unit: 1635
Examiner: --

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Dear Sir:

Pursuant to 37 C.F.R. 1.98, enclosed herewith is a list of documents which the Applicants in the above-identified patent application wish to bring to the attention of the Examiner for consideration in connection with the examination on the merits of this patent application. As some of this information duplicates information presented in related case Serial No. 09/241,561, Applicants have not provided copies of the duplicated documents.

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5,593,974; Rosenberg, et al.; January 14, 1997.

Foreign Patents

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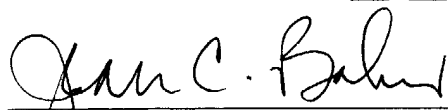
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Respectfully submitted,

Martin G. Sirois, et al.

November 8, 2001

By:



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SIROIS, Martin G. et al.

Filing Date

August 31, 2001

Group Art Unit

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	REF	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
		5,593,974	01/14/97	Rosenberg et al.			01/06/95

FOREIGN PATENT DOCUMENTS

	REF	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation	
							YES	NO
		93/08845	05/13/93	WO				

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		Filing Date August 31, 2001	Group Art Unit

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PDGF-Receptor Tyrosine Kinase Blocker AG1295 Selectively Attenuates Smooth Muscle Cell Growth In Vitro and Reduces Neointimal Formation After Balloon Angioplasty in Swine

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Background—Signaling through protein tyrosine kinases (PTKs) is a major contributor to the transmission of mitogenic stimuli to the interior of the cell and nucleus. The present study was designed to determine the effect of the tyrphostin AG1295, a selective blocker of PDGF-receptor PTK, on the growth of porcine and human smooth muscle cells (SMCs) in culture, on the outgrowth kinetics of SMCs from porcine and human arterial explants, and on neointimal formation after balloon injury in pigs.

Methods and Results—SMCs for culture were obtained from porcine abdominal aortas, human internal mammary arteries, and endarterectomy tissue from a single human carotid artery. Addition of AG1295 to SMCs before PDGF stimulation completely inhibited PDGF- β -receptor tyrosine phosphorylation without affecting the level of PDGF- β -receptor. AG1295 resulted in a selective, reversible inhibition of SMC proliferation in culture (76%) with only mild (13.5%) inhibition of endothelial cell proliferation. The number of SMCs accumulating around explants of porcine carotid arteries and human endarterectomy specimens 12, 15, 19, 22, and 24 days after plating was reduced by 82% to 92% in AG1295-treated compared with nontreated specimens, and initiation of SMC outgrowth was markedly delayed. The numbers of cells accumulated 10 days after initiation of outgrowth were significantly lower in treated versus control explants. Local intravascular delivery of AG1295-impregnated polylactic acid-based nanoparticles (130 ± 25 nm) to the site of balloon injury to porcine femoral arteries resulted in significant reductions in intima/media area ratio and luminal cross-sectional area narrowing by neointima compared with contralateral control arteries to which empty nanoparticles were applied (0.15 ± 0.07 versus 0.09 ± 0.03 , $P = .046$ and $20 \pm 4\%$ versus $10 \pm 4\%$, $P = .0009$, $n = 6$ for both).

Conclusions—The tyrphostin AG1295, a selective blocker of PDGF-receptor kinase, exerts a marked inhibitory effect on the activation, migration, and proliferation of porcine and human SMCs in vitro and an $\approx 50\%$ inhibitory effect on neointimal formation after balloon injury in porcine femoral arteries when delivered via biodegradable nanoparticles. Further studies appear to be warranted to evaluate the applicability of this novel approach to the interventional setting. (*Circulation*. 1998;97:1960-1969.)

Key Words: muscle, smooth ■ tyrosine kinase ■ tyrphostin ■ platelet-derived factors ■ restenosis

Proliferation and migration of activated SMCs, with release of abundant extracellular matrix by these cells, are fundamental to neointimal growth associated with accelerated arteriosclerosis, which continues to plague patients undergoing balloon angioplasty, coronary artery bypass surgery, and heart transplantation. A variety of experimental studies have been directed toward the attenuation of SMCs in vitro and in vivo. Nonetheless, relatively little progress has been made in the development of effective, selective, nontoxic inhibitors of

SMC growth that might eventually be applied in the interventional setting. Recent progress in determining the mechanisms by which growth factors control cell proliferation has contributed to the development of treatment strategies that target specific signal transduction pathways to control proliferative disorders.¹⁻⁵ The binding of specific growth factors with their selective cell surface receptor tyrosine kinases results in its autophosphorylation and activation, leading to downstream signal transduction through chains of intercom-

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Selected Abbreviations and Acronyms

CFA	= common femoral artery
%CSAN-N	= percent luminal cross-sectional area narrowing by neointima
EC	= endothelial cell
ECGS	= endothelial cell growth substitute
IEL	= internal elastic lamina
I/M	= intima to media area
PDGF	= platelet-derived growth factor
PTK	= protein tyrosine kinase
SMC	= smooth muscle cell

municating proteins culminating in cell proliferation.^{6,7} Inhibitors of PTKs have been shown to suppress SMC chemotaxis and proliferation.⁸⁻¹²

The tyrphostins are low-molecular-weight, synthetic compounds whose basic structure can be modified to block specific receptor PTKs or intracellular PTKs.^{3,13} Unlike larger receptor antibodies, the small size of the tyrphostins permits easier access to receptor sites within tissues such as those deep in the media. Recent studies have suggested that the profound selective PTK inhibition of such compounds results from competitive interaction with the ATP-binding domain as well as mixed competitive inhibition with substrate-binding subsites.^{14,15} Development of this class of compounds was based on the concept that it would lead to a more focused control of proliferative disorders, achieve more improved therapeutic indices, and reduce the numerous untoward side effects of the more generalized inhibitors of DNA or RNA synthesis or cytoskeleton-disrupting agents. We recently showed that controlled local delivery of the nonselective PTK blocker AG17 (RG50872) effectively inhibits neointimal formation in a rat carotid artery balloon injury model.¹⁶ The present study takes advantage of the selectivity of the described tyrphostin-type PTK inhibitor.

PDGF, expressed by platelets, SMCs, ECs, and macrophages, has been shown to play an important role in the pathogenesis of injury-induced neointimal formation in the arterial wall, acting as both a mitogen and chemoattractant for SMCs as well as being involved in the transformation of SMCs from their contractile to the proliferative phenotype.^{17,18} In vivo studies have demonstrated that the expression of PDGF ligand and its receptor is elevated after arterial injury.¹⁹ Infusion of PDGF into injured rat carotid arteries and transfection of a plasmid coding for PDGF into pig arteries have also been shown to increase neointimal formation.^{20,21} PDGF receptor levels in SMCs from human atherosclerotic plaques have also been reported to be elevated compared with receptor levels in normal medial SMCs.²² Recently, Sirois et al²³ showed marked upregulation of PDGF receptors after injury to the vessel wall. They have demonstrated that the degree of neointimal formation substantially depends on both PDGF- β -receptor overexpression and its activation by PDGF-BB. They demonstrated further that controlled local delivery of antisense oligonucleotides to PDGF- β receptor reduces neointimal formation in the rat carotid injury model. Finally, PTK blockers of the tyrphostin family have been shown to block PDGF-receptor signal transduction, including

the phosphorylation and activation of phospholipase C- γ , believed to be involved in SMC migration.^{11,12,24,25} We therefore hypothesized that selective blockade of PDGF- β -receptor activation should also result in marked inhibition of SMC activation, migration, and proliferation.

We show here that the tyrphostin AG1295, a selective blocker of PDGF-receptor PTK, inhibits PDGF-BB-induced PDGF- β -receptor phosphorylation without affecting receptor protein levels, selectively inhibits porcine and human SMC proliferation in culture with only a minimal effect on ECs, attenuates the outgrowth of SMCs from porcine and human arterial explant tissue in vitro, and inhibits neointimal formation after balloon injury in pigs by $\approx 50\%$ after local, controlled, intravascular delivery of biodegradable nanoparticles.

Methods

Effect of AG1295 on PDGF-Induced Receptor Autophosphorylation in Intact Cells

Subconfluent porcine arterial SMCs cultivated in DMEM supplemented with 15% FCS were synchronized for 20 hours in medium containing 2% FCS. After preincubation with AG1295 (10 $\mu\text{mol/L}$) for 60 minutes and with Na_2VO_4 (100 $\mu\text{mol/L}$) for 5 minutes, the cells were stimulated with PDGF-BB (100 ng/mL) for 10 minutes at 37°C. The cells were solubilized in NP-40 (1% lysis buffer). The analysis of PDGF- β -receptor phosphorylation was performed as described previously.²⁶ Briefly, cell lysates were used for immunoprecipitation with the PDGF- β -receptor-specific antiserum R3, and the samples were subjected to SDS-PAGE (7.5%) for receptor analysis and blotted onto a nitrocellulose membrane (Hybond C extra, Amersham). Phosphorylated proteins were detected by immunoblotting with the horseradish peroxidase-conjugated phosphotyrosine antibody RC20H (Transduction Laboratories), followed by application of a chemoluminescence-based detection system (ECL, Amersham) and autoradiography. Detection of receptor proteins was performed in a similar way by immunoblotting with the specific R3 antiserum followed by several washing steps and the application of a horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham) and visualization with chemoluminescence and autoradiography as described above.

Cell Culture Techniques

SMCs were obtained under aseptic conditions from 6 pig abdominal aortas, 6 human internal mammary arteries, and endarterectomy tissue from a single human carotid artery by the explant technique.²⁷⁻²⁹ Specimens from the operating room were transferred on ice to the tissue culture room, where each artery was cut open and the endothelial surface mechanically scraped. The vessels were then cut into 2-mm² fragments, which were placed in culture dishes with DMEM supplemented with 15% (vol/vol) FCS, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 0.2 mol/L L-glutamine. The medial tissue fragments were then placed in an incubator at 37°C in 9% CO_2 until SMC outgrowth was detected. Uniform populations of SMCs that displayed the characteristic "hill-and-valley" growth pattern were subcultured with 0.25% trypsin. For experiments testing the effect of AG1295 on growth inhibition and reversibility, SMCs from passages 1 to 3 were replated on 15-mm wells pretreated with 3 $\mu\text{g/cm}^2$ fibronectin^{30,31} (Biological Industries) at 15 000 cells/well.

ECs were isolated from porcine carotid arteries.^{32,33} Under aseptic conditions, both common carotid arteries were isolated, and the distal end of each artery was cannulated through an arteriotomy and ligated. The arteries were then perfused with PBS, and the proximal end was ligated, isolating a 5- to 7-cm-long blood-free portion of the artery. The isolated portion of each artery was filled with PBS containing calcium and magnesium with 0.1% collagenase. The segments were excised and incubated for 10 minutes at 37°C in

sterile bottles containing PBS. The arterial effluent was then flushed out with medium (M199 supplemented with 15% FCS, penicillin 100 U/mL, streptomycin 100 μ g/mL, 0.2 mol/L L-glutamine, and 25 μ g/mL ECGS [Biomedical Technologies, Inc]) and collected in 50-mL centrifugation tubes containing 5 mL of medium. The cell suspension was centrifuged (200g, 5 minutes) and the pellet resuspended in culture medium. Cells were seeded on fibronectin-coated dishes at a seeding density of 15 000 cells/well and incubated at 37°C in 9% CO₂. The ECGS (25 μ g/mL) was added every other day until confluence. At confluence, the cells were removed with trypsin-EDTA solution (0.25% trypsin plus EDTA 1:2000 in Puck's saline), resuspended in culture medium, counted, and replated at 15 000 cells/well in fibronectin-coated four-well dishes (15 mm) for the growth inhibition experiments.

Inhibition of Cell Proliferation and Reversibility

Monolayer cell growth inhibition and reversibility experiments were repeated three or four times, with each experiment having been performed in triplicate. Approximately 15 000 cells (SMCs or ECs) in 1 mL of culture medium supplemented with 15% FCS were seeded on day 0 in 15-mm wells precoated with fibronectin. Cultures were treated with AG1295 (10 μ mol/L) dissolved in 0.1% DMSO on days 1 and 3. On day 6, cultures were washed and the cells allowed to recover. Cells were counted on days 3 and 5 for inhibition and on days 7, 10, and 15 for reversibility. The medium supplemented with serum (M199 with ECGS for ECs and DMEM for SMCs) was changed every other day. The effect of AG1295 on cell proliferation was compared with three control groups: (1) DMSO (0.1%) without AG1295; (2) medium with serum only; and (3) AG17 (10 μ mol/L), a potent, nonselective PTK blocker.^{5,34-36}

Arterial Explant Techniques

Explant tissue was obtained from six porcine common carotid arteries and human atheroma retrieved from a single patient undergoing carotid endarterectomy. The specimens were placed in PBS with penicillin (100 U/mL)/streptomycin (100 μ g/mL). After a washing in additional PBS, the endothelium was removed by gentle scraping, and the adventitia was peeled off with fine forceps. The medial specimens were cut into 1-mm² fragments with a sharp scalpel blade. To measure cellular accumulation around explants, four fragments were placed in four-well dishes pretreated with fibronectin. For outgrowth initiation and outgrowth index studies, 96-well plastic culture dishes were pretreated with fibronectin, and an individual fragment was placed in each well. Explants were left undisturbed for 45 minutes without growth medium at room temperature to allow for explant attachment. Fragments in the four-well plates were then immersed in 1 mL of culture medium supplemented with 7.5% human serum and 7.5% FCS (human endarterectomy specimens) or 15% FCS (porcine arterial specimens). Fragments in 96-well plates were immersed in 150 μ L of the appropriate culture medium. The plates were placed in a humidified incubator (5% CO₂) at 37°C, and the medium was changed every 2 days. AG1295 (50 μ mol/L dissolved in 0.5% DMSO) was added to the culture medium of treated explants every 2 days throughout the experiment. An equal concentration of DMSO was added to the medium of control explants. Samples of arterial explants for histological evaluation were taken before and immediately after removal of the endothelium. These samples were fixed in 4% buffered formaldehyde, dehydrated in ethanol and xylene, and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin-eosin and by the Movat technique.³⁷

The overall accumulation of SMCs around each explant was measured at 12, 15, 19, 22, and 24 days after plating. Experiments were performed in triplicate so that for each of the five time periods tested there were three wells containing four explants, each yielding 12 treated and 12 control explants, for a total of 120 explants. Each experiment was repeated twice. At each of the five time periods after plating, the explants were removed from the wells, and the cells that had grown out from the tissue and accumulated on the plate were enzymatically dispersed (0.25% trypsin, 1 mmol/L EDTA) and

counted in a Coulter Counter. The accumulation of SMCs around the explants was expressed as the total number of cells per well.

The time course of the initiation of outgrowth was determined with 95 additional control and 96 treated porcine carotid explants and 23 control and 23 treated explants of human atheroma specimens (all from the same individual). The specimens were scored every other day by two independent observers to determine the number of explants yielding outgrowth of SMCs. Explants showing two or more SMCs at the edges of the tissue were counted as positive for outgrowth initiation. The time course of outgrowth (percentage of explants yielding outgrowth per days in culture) was plotted. The porcine carotid and human endarterectomy explants were followed until the proportion of explants with outgrowth was constant (21 and 34 days, respectively).

To establish the rate of proliferation of SMCs after their activation in control versus treated explants, an index of outgrowth was determined by counting the number of SMCs that had grown around each explant 10 days after outgrowth was first observed. This index excludes the lag time before outgrowth initiation and thereby permits the estimation of the overall rate of SMC proliferation in cells already activated. The cells that had proliferated around each explant at this time were enzymatically dispersed, pooled, and counted.

Immunocytochemistry

Identification of SMCs in the outgrowth phase and in the first subculture was confirmed by α -actin staining of primary outgrowth and passaged cells. The cells were fixed in 4% paraformaldehyde and immunostained with mouse monoclonal antibodies directed against α -smooth muscle actin (Mouse Monoclonal, Sigma Chemical Co, product No. 6582, clone 1A4). The secondary antibody, peroxidase-conjugated Affinipure goat anti-mouse IgG (heavy and light chains) (Jackson Immuno Research Laboratories), was visualized by incubation with an AEC chromagen (peroxidase chromagen C3-amino,9-ethyl carbazole; Biomeda Corp).

Neointimal Formation After Balloon Injury In Vivo

Eight juvenile domestic swine (15 to 20 kg) were sedated by intramuscular injection of 1% propionylpromazine (0.1 mL/kg). Anesthesia was induced with ketamine hydrochloride (20 mg/kg IM) and droperidol (0.2 mg/kg IM) followed by 6% sodium pentobarbital (0.25 mL/kg IV). After endotracheal intubation, the pigs were ventilated with a mixture of oxygen and room air. After surgical exposure of the CFAs and the proximal portions of the superficial femoral arteries bilaterally, all side branches of the CFA were ligated. After administration of heparin (5000 U IV bolus), a high-torque floppy angioplasty guidewire (0.014 in) was inserted into the CFA through an arteriotomy in the superficial femoral artery, followed by over-the-wire passage of the balloon angioplasty catheter (3.0 to 3.5 mm in diameter, noncompliant, 20 mm long, balloon-to-artery ratio, 1.5:1). The balloon was then inflated in the CFA and withdrawn under tension (7 to 8 atm). After five passes, the balloon was kept inflated in the CFA for 2 minutes. All balloon injuries were performed by the same investigator. After deflation, the balloon was removed, an infusion catheter was inserted over the wire into the CFA, and the wire was removed. The injured segment of the CFA was isolated by proximal and distal occlusion with Yasargil atraumatic arterial clips. Polylactic acid-based nanoparticles (130 \pm 25 nm), prepared by emulsification evaporation, with or without AG1295 (90 to 110 μ g/mL) were delivered into the isolated injured segment (0.3- to 0.4-mL volume). The solution was retained within the isolated segment for 30 minutes (AG1295-impregnated ipsilaterally or bare nanoparticles contralaterally). After withdrawal of the solution, the clamps and infusion catheter were removed, flow was restored, and the superficial femoral artery was tied. The presence of nanoparticles within the arterial wall after this procedure was confirmed in two additional arteries by high-performance liquid chromatography 24 hours after restoration of blood flow. After closure of the skin, the animals were allowed to recover and were returned to their pens. One animal was found dead in its pen after the

surgery, and a second was excluded because of surgical mishap on the sham-operated control side. Four weeks later, under general anesthesia and mechanical ventilation, both femoral arteries were exposed at the site of balloon injury. The abdominal aorta and inferior vena cava were isolated, ligated, and cannulated at the level of the renal vessels. The animals were euthanized with sodium pentobarbital (60 mg/kg) followed by a rapid bolus of KCl (40 mEq/L IV). The arteries were flushed via the aortic cannula with normal saline (1000 mL with 3 mL heparin [5000 U/mL], 37°C, 90 mm Hg) and pressure-perfused with 4% buffered formaldehyde (1000 mL, 37°C, 90 mm Hg). The perfusion effluent was drained via the inferior vena cava cannula. Segments of the right and left CFAs were excised, cut into 1- to 2-mm segments, and embedded in paraffin. Cross sections 4 μ m thick were stained by the Movat pentachrome technique. Computerized morphometric analysis was performed on all sections with a CUE-2 image analyzer (Galai Productions, Ltd) in association with an Olympus BH-2 microprojection system. The areas measured were total area bounded by the external elastic lamina (EEL area), area bounded by the IEL (IEL area), and area occupied by the lumen (LU area). Derived measurements of neointimal formation included the I/M ratio (IEL area-LU area \div EEL area-IEL area) and the %CSAN-N [(IEL area-LU area) \times 100 \div IEL area].

Statistical Analysis

Results are expressed as mean \pm SD. For the in vitro studies, the effect of the various doses of tyrphostins versus control, at any time period, was assessed by one-way ANOVA with Fisher's protected least significant difference as the post hoc test. Comparisons between tyrphostin treatment and control for individual morphological parameters at one specific time point were assessed by unpaired, two-tailed *t* test. Comparisons between tyrphostin treatment and control for individual morphological parameters over multiple time points were assessed by two-factor ANOVA. Histomorphometric comparisons were made on the section most narrowed by neointima from each artery. The differences in I/M ratio and %CSAN-N after balloon injury in vivo between AG1295-treated and contralateral sham control arteries were determined by the paired two-tailed *t* test. The Statview II statistical package (Brain Power, Inc) was used for these calculations.

Results

Inhibition of PDGF-Induced Receptor Autophosphorylation

Stimulation of porcine arterial SMCs with PDGF-BB (100 ng/mL) resulted in strong phosphorylation of the PDGF- β -receptor on tyrosine residues. Addition of AG1295 to the cells before PDGF stimulation completely inhibited PDGF- β -receptor tyrosine phosphorylation (Fig 1A). AG1295 did not affect the level of PDGF- β -receptor protein present in the cells (Fig 1B).

Cell Culture Studies of Enzymatically Dispersed Cells

Porcine Aortic SMCs

Treatment with AG1295 resulted in a 46% mean reduction in SMC count by day 3 compared with DMSO-treated control cells and a 76 \pm 2% (mean \pm SD) reduction over control by day 5. The nonselective PTK blocker AG17 inhibited SMC growth by 79% and 91 \pm 2% at days 3 and 5, respectively. Whereas the effect of AG17 was not reversible and cells did not resume proliferation after treatment was withdrawn, the inhibitory effect of AG1295 was completely reversible (Fig 2).

PDGF-BB
AG1295

A

220 kDa -

97 kDa -

66 kDa -

B

220 kDa -

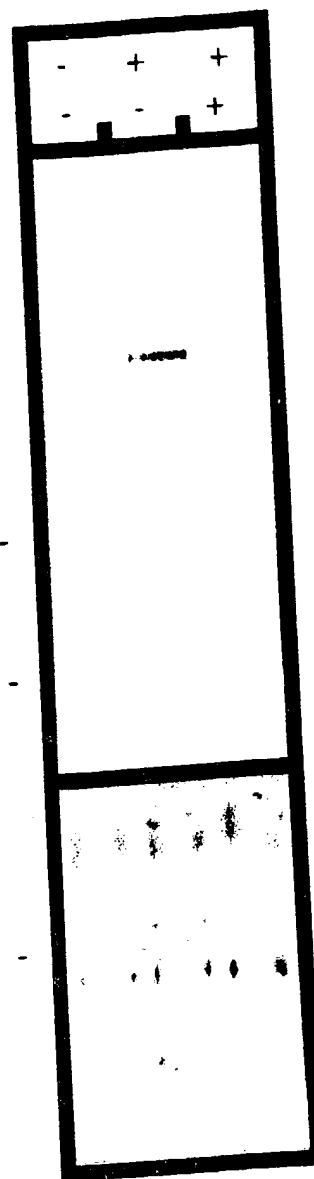


Figure 1. Immunoblot analysis. Effect of AG1295 on PDGF-BB-induced tyrosine phosphorylation of PDGF- β -receptor in intact porcine arterial SMCs (A) as well as levels of PDGF- β -receptor expression (B). Pretreatment and stimulation of porcine arterial SMCs was followed by immunoprecipitation of PDGF- β -receptor (R3 antiserum), SDS-PAGE (7.5%), and immunoblot analysis (RC20H in A; R3 in B). Detection of hybridizing antibodies was obtained with a chemoluminescence-based detection system (ECL, Amersham).

Porcine ECs

The inhibitory effect of AG1295 on EC proliferation was minimal, resulting in only a 10% mean reduction of cell growth by day 3 and a 13.5 \pm 3% reduction by day 5 compared with control ECs (Fig 3). This mild inhibitory effect was completely reversible. The nonselective AG17 resulted in a 55% and 91 \pm 12% mean reduction of EC growth by days 3 and 5, respectively (Fig 3), and this effect was not reversible after treatment was discontinued.

Human Internal Mammary Artery SMCs

Treatment with AG1295 resulted in a 50% mean reduction in SMC proliferation by day 3 and a 72% mean reduction by day

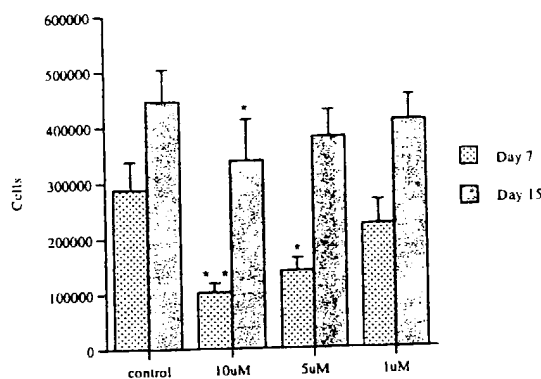


Figure 2. Dose response and reversibility of inhibitory effect of AG1295 on porcine SMC proliferation. For this experiment, cells were treated with AG1295 on days 1 and 3. On day 7, cultures were washed and cells allowed to recover. Cells were counted on day 7 for inhibitory effect and on day 15 for recovery. AG1295 1, 5, and 10 $\mu\text{mol/L}$ caused 23%, 51%, and 64% inhibition of proliferation, respectively. In all doses tested, effect was not toxic and reversible. * $P < .05$, ** $P < .001$ vs control by one-way ANOVA.

5 compared with untreated or DMSO-treated cells. This effect was completely reversible (Fig 4A).

Human Atheroma-Derived SMCs

AG1295 inhibited human atheroma-derived SMC growth by 64% and 74% by day 3 and 5, respectively, compared with untreated or DMSO-treated cells. This effect was completely reversible (Fig 4B).

Arterial Explant Studies

Outgrowth from porcine carotid artery and human carotid endarterectomy explants began at the margins of the specimens 4 and 8 days after plating, respectively. The first cells that migrated out of the margins of the explanted tissue were morphologically and immunohistochemically indistinguishable between treated and control wells. These cells were elongated and spindle-shaped, and only a small percentage (1% to 5%) stained positive for filamentous α -actin within the first 5 days after outgrowth initiation in both treated and

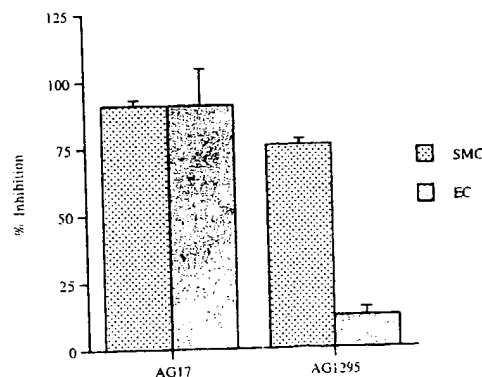


Figure 3. Degree of inhibition of cell proliferation by AG17 and AG1295. Note that AG17, the nonselective PTK blocker, inhibited both SMC and EC proliferation (mean, 91% inhibition for both ECs and SMCs), but AG1295 selectively inhibited SMC proliferation (mean, 76% inhibition) with only a mild inhibitory effect on EC proliferation (mean, 13.5%). Cells were treated with tyrophostins on days 1 and 3 and counted on day 5.

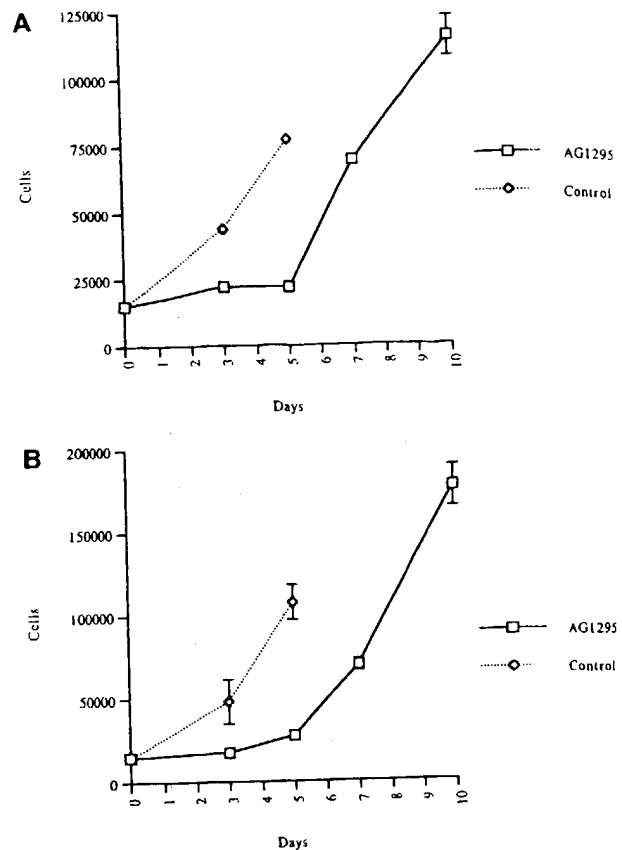


Figure 4. Inhibitory effect of AG1295 on human internal mammary artery (A) and human atheroma-derived SMC (B) proliferation and reversibility of the effect. Mean inhibitory effect during treatment days 3 and 5 was 64% and 74%, respectively, for human atheroma-derived cells and 50% and 72% for human internal mammary artery-derived cells. $P < .0001$ and $P < .003$, respectively, by two-way ANOVA.

control explants. These cells assumed the well-known hill-and-valley configuration often attributed to the proliferative phenotype of SMCs in culture. However, after reaching confluence (≈ 10 days after outgrowth initiation), the cells appeared to redifferentiate, and the percentage of SMCs that stained positive for filamentous α -actin reached 50% to 70%. In general, the more distant cells from the explant exhibited more intense α -actin staining in both treated and control specimens. A much greater percentage of SMCs from AG1295-treated explants (versus control) assumed the larger, polygonal, and well-spread profile with numerous α -actin-positive stress fibers often attributed to the contractile phenotype. The morphological features of SMCs from AG1295-treated explants were similar to those of SMCs seen in monolayers of passaged cells (Fig 5). The explanted tissue in the control wells appeared to shrink as the cells migrated out of the explant, but shrinkage was not apparent in the treated tissue.

Histological evaluation of the hematoxylin-eosin-stained and Movat-stained sections of the explants that underwent luminal scraping showed complete desquamation of the endothelium with the virtual absence of the IEL. The IEL was intact in explants in which the endothelium had not been scraped off.



Figure 5. SMC accumulation at margins of porcine carotid explants 5 days after outgrowth initiation. Note that SMCs growing out from control explants (top) appear more numerous and are more spindle-shaped, with fusiform nuclei and less prominent α -actin staining. These cells are rapidly proliferating. Fewer cells are seen around explants treated with tyrphostin AG1295 (middle). These SMCs appear larger, with a more spread, polygonal morphology and intense α -actin-positive fibers ($\times 600$). Bottom, Pig SMCs treated with AG1295 ($\times 1200$).

Outgrowth Kinetics From Explant Tissue

Initiation of Outgrowth

Treating porcine carotid explants with AG1295 resulted in a marked prolongation of the time between the plating of the arterial tissue and the appearance of cells around the explants. In control explants, 36% of the 95 explants showed SMC growth initiation 7 days after plating. In contrast, in the tyrphostin-treated specimens, only 12% of the 96 explants had cells at their margins at this time. Outgrowth was seen in 50% of control specimens by day 8, whereas outgrowth in

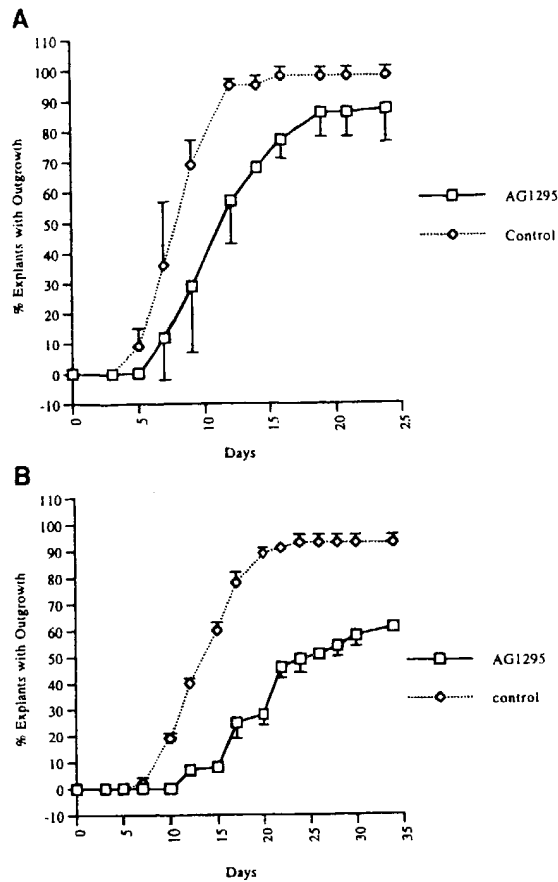


Figure 6. Delay in outgrowth initiation of SMCs caused by AG1295 from porcine arterial explants (A) ($P < .05$) and from human atheroma explants (B) ($P < .0001$) (two-way ANOVA).

50% of the treated specimens was observed only on day 12. The outgrowth reached a plateau on day 12 in the control tissue (96% to 100%) but not until day 19 in the treated samples (91% to 95%) (Fig 6A).

The human carotid endarterectomy specimens showed similar outgrowth kinetics and response to tyrphostin treatment. Seventeen percent of control explants showed SMC outgrowth initiation at day 10, but at this time, none of the treated specimens had outgrowth (Fig 6B). Likewise, by day 14, 50% of control explants had outgrowth, but in tyrphostin-treated specimens, 50% outgrowth was seen only after day 23. As with the porcine specimens, a delay in the time to reach the plateau was also found in the human tissue. In the untreated human control specimens, SMC outgrowth became constant at 91% by day 22, but in the treated specimens, this plateau was reached only at day 30, and the percentage of specimens showing outgrowth at plateau was only 61%.

Outgrowth Index

The mean number of SMCs that accumulated around porcine carotid explants 10 days after outgrowth initiation was first observed was 70% lower in those treated with AG1295 than in control samples (8489 ± 1764 versus $28\,626 \pm 2977$, $P < .00001$) (Fig 7A). Likewise, the mean SMC accumulation 10 days after onset of outgrowth from human atheroma specimens was significantly lower in explants treated with AG1295 than in control samples (6937 ± 704 versus

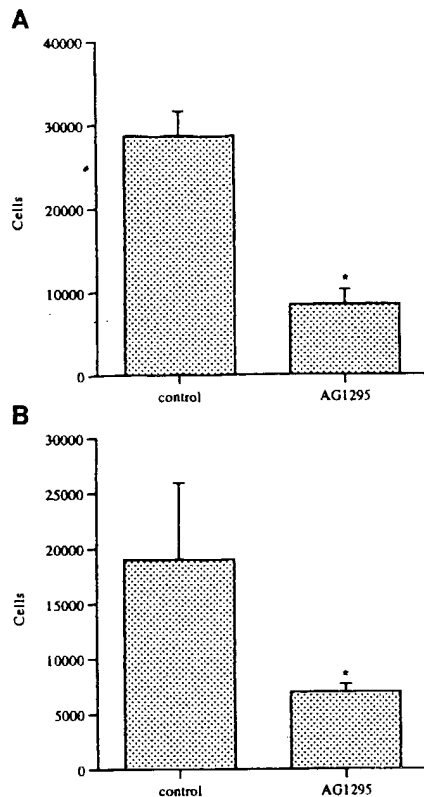


Figure 7. Outgrowth index of SMCs from porcine carotid artery explants (A) and human endarterectomy specimens (B). Note marked inhibitory effect of AG1295 on SMC outgrowth and accumulation around explants 10 days after initiation of outgrowth in each explant. * $P < .001$ by unpaired, two-tailed t test.

$18,945 \pm 6,943$, $P = .0001$) (Fig 7B). This represents a 63% inhibition of SMC accumulation around the treated explants.

Overall Accumulation of SMCs Around Porcine Carotid Explants

The total number of cells accumulating around control explants increased with time. However, in explants treated with AG1295, SMC growth was less by 82% to 92% at all times (Figs 8 and 9).

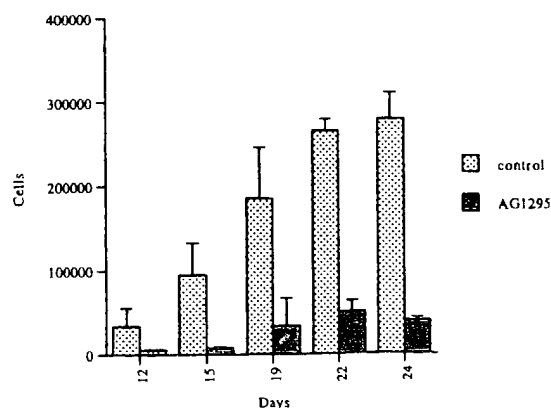


Figure 8. Inhibitory effect of AG1295 on SMC accumulation around porcine explants measured 12, 15, 19, 22, and 24 days after plating (total number of cells per well). Inhibition of cell accumulation (AG1295-treated vs control) was between 82% and 92% at all time points ($P < .0001$ by two-way ANOVA).

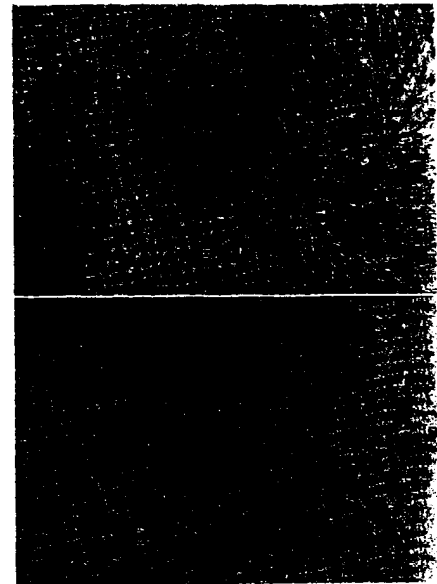


Figure 9. Porcine arterial SMC accumulation around carotid arterial explants (24 days after plating of explant). Note significantly fewer cells growing out of AG1295-treated explant (top) vs control explant (bottom) (see Fig 8). Morphological differences between treated and untreated cells are seen in higher magnification in Fig 5. E indicates site of explant that was removed before photography. α -Actin stain ($\times 300$).

Neointimal Formation After Balloon Injury In Vivo

Local intravascular delivery of AG1295-impregnated polylactic acid-based nanoparticles to the site of controlled balloon injury to porcine femoral arteries resulted in a significant reduction in I/M ratio compared with contralateral control arteries to which empty nanoparticles were applied (0.15 ± 0.07 versus 0.09 ± 0.03 , $P = .046$, $n = 6$ for both) (Fig 10). The utility of the I/M ratio for assessing neointimal narrowing across arterial samples presupposes consistency in medial area and overall vessel wall size. Whereas the medial areas were very consistent ($176 \pm 14 \times 10^4 \mu m^2$), the overall vessel wall size showed a slightly greater variability ($313 \pm 60 \times 10^4 \mu m^2$). For this reason, %CSAN-N was also used (see "Methods"), which measures the degree to which the IEL area is reduced by neointima and normalizes, to a great degree, the effect of changes in vessel wall size. The mean %CSAN-N at sites of balloon injury of arteries to which AG1295-impregnated nanoparticles were delivered was significantly less than contralateral control arteries ($10 \pm 4\%$ versus $20 \pm 4\%$, $P = .0009$). Inflammatory cell infiltrate within the intima and media at sites of nanoparticle delivery was relatively light or nondetectable, and no difference was detected between AG1295-impregnated and empty nanoparticles. Inflammatory cell infiltrate within the adventitia did not appear to depart from that seen in angioplastied porcine arteries not subjected to this intravascular delivery.

Discussion

These experiments demonstrate that tyrophostin-mediated inhibition of the PDGF- β -receptor autophosphorylation results in the selective inhibition of SMC activation, proliferation, and migration in vitro, with a minimal effect on ECs and a

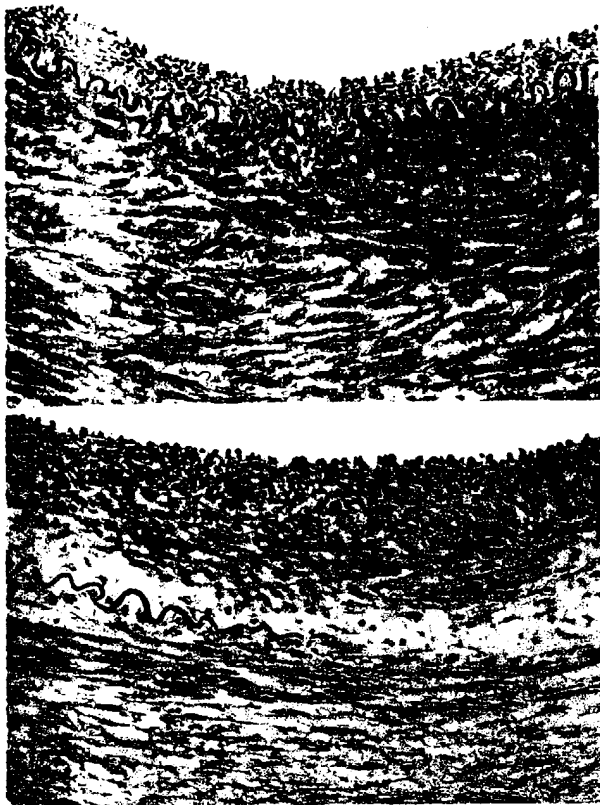


Figure 10. Porcine femoral arteries 28 days after balloon injury showing marked reduction in neointimal area after intravascular delivery of AG1295-impregnated nanoparticles (top) compared with sham-operated control artery (bottom) that received empty nanoparticles. Movat stain ($\times 220$).

significant reduction of neointimal formation *in vivo* in a pig balloon injury model. The tyrphostin AG1295 completely inhibited the PDGF-BB-induced phosphorylation of the PDGF- β -receptor tyrosine residues of porcine arterial SMCs without affecting the level of PDGF- β -receptor protein present in these cells, providing additional support of effective inhibitory activity without significant toxicity. Although it has been shown that this tyrphostin is a highly selective blocker of the PDGF-receptor PTK,¹⁵ given the large number of known protein kinases, the possibility that AG1295 may display some activity against other kinases cannot be excluded.

Because *in vitro* findings from passaged SMCs may be too far removed from the *in vivo* situation to reflect the biological properties of SMCs in the vessel wall, we used the arterial explant model as a "bridge" between the *in vitro* cell culture experiments and the *in vivo* porcine balloon injury experiments. The explant model permits the *ex vivo* study of SMC transformation, migration, and proliferation in a system that preserves many but certainly not all aspects of the arterial tissue relationships and microenvironment, including the variety of local paracrine and autocrine systems.³⁸ Outgrowth initiation is the first end point in this model. The time for the first cell to appear at the margins of the explant is a marker for tissue activation, because it represents the first appearance of a transformed population of cells able to migrate out of the tissue and proliferate. The second end point is the accumu-

lation of cells around each explant 10 days after the appearance of the first cell. This end point controls for variations in the lag time for activation in each explant. With this model, AG1295 markedly reduced the total number of cells accumulating around the explanted porcine and human arterial specimens, prolonged the time to initiation of outgrowth, and delayed the time to reach the growth plateau. The outgrowth index, calculated from the total number of cells present 10 days after the initiation of outgrowth for each specimen, a parameter that spans the logarithmic phase of SMC proliferation, was also markedly reduced.

The determination of the overall number of SMCs accumulating around the explants at various time points after plating does not discern the relative role of activation, migration, and/or proliferation but is a straightforward method of assessing the combined effects of these important cellular events. The effects of AG1295 on SMC activation and proliferation were more specifically observed from the significant delay in the initiation of SMC outgrowth from the explants, the prolongation of time to attainment of the growth plateau, and the marked effect on the outgrowth index. The effect of this agent was also apparent from the morphological and immunohistochemical observations showing that AG1295 seemed to maintain the SMCs in a contractile and predominantly nonproliferating phenotype even though the tissue was subjected to explantation and exposed to serum mitogens.

The marked inhibitory effect of AG1295 on SMCs *in vitro* was confirmed *in vivo* by intravascular delivery of tyrphostin-impregnated biodegradable nanoparticles to the site of balloon angioplasty in porcine femoral arteries. The vast majority of cells in the media of healthy, uninjured adult arteries are SMCs. Injury to the vessel wall, with loss or damage to the endothelium, causes a subpopulation of the quiescent, differentiated SMCs to lose their contractile myofibrillar apparatus and transform into synthetic cells with large amounts of rough endoplasmic reticulum, ribosomes, and mitochondria. This transformation, directed at least in part by PDGF, is associated with SMC migration and proliferation followed by elaboration of abundant extracellular matrix. The signal transduction induced by PDGF-BB, considered by many to be the strongest known mitogen and chemoattractant for arterial SMCs, stimulates directed migration and proliferation of arterial SMCs into the neointima after arterial injury.³⁹ It has been suggested that if the endothelium regenerates rapidly after injury, the synthetic SMCs return to a contractile, nondividing phenotype, and neointimal formation is reduced.⁴⁰ If, however, the injury is severe or sustained, the cells may remain in their synthetic-proliferative phenotype and retain their heightened responsiveness to mitogens. PDGF receptors are thought to be expressed primarily in SMCs, whereas vascular endothelial growth factor receptor expression is considered to be restricted largely to the endothelium. The novel approach to the inhibition of neointimal formation by AG1295 presented in the present study takes advantage of the marked selective, nontoxic inhibition of SMC PDGF- β -receptor kinase, with virtually no effect on the kinase activity of the vascular endothelial growth factor receptor.¹² By a different methodological approach, Chang et al⁴¹ recently reported inhibition

of SMC proliferation in vitro and in vivo in a rat carotid artery model of balloon injury by adenovirus-mediated overexpression of the cyclin-dependent kinase inhibitor p21, which blocks the initiation of the S phase of the cell cycle and inhibits proliferating cell nuclear antigen, but further studies are necessary to determine the selectivity of this regimen across cell types and species. Recent experiments using antisense oligonucleotides and neutralizing antibodies further support the concept of PDGF- β -receptor blockade as a treatment strategy to inhibit neointimal formation and premature arterial stenosis.⁴² The small molecular size of the tyrphostins, such as AG1295, used in the present study has the additional advantage of permitting easier access to SMC-PDGF receptors within the media and adventitia, and the use of small, biodegradable nanoparticles as the delivery vehicle provides for prolonged intramural exposure to the site of injury.

In conclusion, these studies demonstrate a profound effect of the tyrphostin AG1295 on the outgrowth kinetics of SMCs in culture and explant tissue and a marked inhibitory effect on neointimal formation after balloon injury in vivo. On the basis of these results, additional studies appear to be warranted to determine the long-term effects of intravascular delivery of tyrphostin-impregnated biodegradable nanoparticles on the arterial wall to evaluate the applicability of this novel approach to the interventional setting.

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Basic Science Reports

Restenosis Following Angioplasty in the Swine Coronary Artery Is Inhibited By an Orally Active PDGF-Receptor Tyrosine Kinase Inhibitor, RPR101511A

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Background—Platelet-derived growth factor (PDGF), a purported mediator of arterial response to injury, stimulates proliferation, chemotaxis, and matrix production by activation of its membrane receptor tyrosine kinase. Because these activities underlie restenosis, inhibition of the PDGF-receptor tyrosine kinase (PDGFr-TK) is postulated to decrease restenosis.

Methods and Results—RPR101511A is a novel compound which selectively and potently inhibits the cell-free and in situ PDGFr-TK and PDGFr-dependent proliferation and chemotaxis in vascular smooth muscle cells (VSMC). To evaluate the effect of RPR101511A ($30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ BID for 28 days following PTCA) on coronary restenosis, PTCA was performed in hypercholesterolemic minipigs whose left anterior descending (LAD) coronary artery had been injured by overdilation and denudation, yielding a previously existing lesion. Angiographically determined prePTCA minimal lumen diameters (MLD) were similar in vehicle and RPR101511A-treated pigs (1.98 ± 0.09 versus 2.01 ± 0.08 mm) and increased to the same extent in the 2 groups following successful PTCA (2.30 ± 0.06 versus 2.52 ± 0.13). At termination, there was an average 50% loss of gain in the vehicle-treated group but no loss of gain with RPR101511A (2.16 ± 0.05 versus 2.59 ± 0.11 , $P < 0.001$). Morphometric analysis of the LAD showed that RPR101511A caused a significant decrease in total intimal/medial ratio (0.96 ± 0.58 versus 0.67 ± 0.09 , $P < 0.05$).

Conclusions—RPR101511A, which acts by inhibition of the PDGFr-TK, completely prevented angiographic loss of gain following PTCA and significantly reduced histological intimal hyperplasia. (*Circulation*. 1999;99:3292-3299.)

Key Words: angioplasty ■ restenosis ■ platelet-derived factors

A clear understanding of the phenomenon of restenosis following angioplasty remains elusive. Restenosis following angioplasty occurs as a consequence of catheter-induced SMC migration, proliferation, matrix production, vasospasm, and remodeling,^{1,2} events driven by inappropriate growth factor ligand and receptor expression.^{1,2} Platelet-derived growth factor (PDGF) is one of several growth factors implicated in the restenotic process.³ PDGF ligands, established as potent mitogens, chemotactic agents, and inducers of matrix synthesis, produce effects by dimerization and activation of PDGF-receptor (PDGFr) subunits.⁴ Furthermore, as an essential co-stimulator in biologic responses of other cytokines/growth factors and mediators of the coagulation cascade,^{5,6} PDGF has an extensive biological scope of action.

In vivo, in small animal models of vascular injury, PDGF acts primarily to induce SMC migration^{7,8} and secondarily to promote intimal proliferation.^{8,9} Additionally, PDGFr activity, as determined by the state of receptor autophosphorylation, increases several days postinjury and persists for several weeks,^{10,11} providing a functional link between expression of PDGFr/ligand mRNA⁹ and proteins^{10,11} and potential biological influence on arterial stenosis. Results of immunocytochemical and in situ hybridization studies of human restenotic lesions^{12,13} reveal the presence of PDGF-A and -B ligands and PDGFr from 6 to 56 days following PTCA and the correspondent absence of proteins and mRNA in nonlesioned sites. The expression of both ligands and receptor in regions of vascular repair provides evidence that autocrine/paracrine

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loops promoting PDGF-driven cellular activities, occur in human restenotic lesions.^{12,13}

With evidence supporting a critical role for PDGF in arterial repair and restenosis following angioplasty, we investigated the ability of a selective inhibitor of the PDGFR-tyrosine kinase (TK) to block restenosis following angioplasty. We demonstrate that RPR101511A, a novel inhibitor of PDGFR-TK, abolishes PDGF-dependent cellular activities *in vitro* and when administered orally to the hypercholesterolemic adult minipig significantly reduces restenosis following angioplasty.

Methods

In Vitro Assays

The effect of RPR101511A on PDGFR-TK was evaluated using the immunoprecipitated PDGFR cell-free ELISA assay¹⁴; the PDGFR was purified from human VSMC and the final ATP concentration was 20 μ mol/L. Inhibition of PDGFR-dependent activities, including *in situ* receptor autophosphorylation, mitogenesis, cell number, and viability were as described.¹⁵ Exceptions were the use of human aortic VSMC (passage 4 to 6) and swine coronary VSMC. The latter were obtained by dispersal from slaughterhouse coronaries (passage 1 to 3). VSMC were growth-arrested for 24 hours, pretreated for 30 minutes with RPR101511A, and stimulated with a submaximal dose of PDGF-BB (10 ng/mL) or recombinant PDGF-AA (40 ng/mL). For western analysis, chemiluminescence was used to quantify antiphosphotyrosine and anti-PDGFR-detected proteins.

PDGFR-dependent chemotaxis was evaluated in a 96-well modified Boyden chamber (Neuroprobe, Inc) containing a collagen-coated polycarbonate membrane with cells preloaded with the fluorescent probe, calcein AM (5 μ mol/L). PDGF (3 ng/mL) was placed in the lower chamber, and cell migration in the presence and absence of RPR101511A after 4 hours of incubation was determined by quantitation of fluorescence associated with the pores and underside of the membrane with Cytofluor II at excitation/emission wavelengths of 485/530.

To determine the effect of RPR101511A on other kinases, published protocols for CSF-1-receptor¹⁶ and EGF-receptor¹⁷ were followed. Protein kinase C (PKC) and protein kinase A (PKA) assays were conducted using commercially available kits according to the manufacturer's instructions (Sigma).

Measurement of coronary artery vasoreactivity followed published methods.¹⁸ Swine coronary artery rings were treated with RPR101511A, and steady-state tension produced by escalating concentrations of PDGF-BB, serotonin, or angiotensin II were recorded.

Restenosis Model

Thirty adult male Yucatan minipigs (Charles River Labs, Wilmington, Mass), weighing 16 to 30 kg, were housed individually at Rhone-Poulenc Rorer (RPR), Collegeville, Pa. Surgical and experimental procedures were performed according to a protocol approved by the Animal Care and Use Committee, RPR.

For lesion creation, pigs were anesthetized with Telazol (4.4 mg/kg, IM), intubated and ventilated with 2% isoflurane-oxygen. The medial ear vein and right external jugular vein were cannulated for administration of lactated Ringer's solution, heparin (3000 U bolus followed by 1000 U every 20 minutes), nitroglycerin (120 μ g/min), and lidocaine (1 mg/min). An 8F introducer sheath was placed in the right carotid artery. Using fluoroscopic-guided assistance (Stenoscopy II, GE Medical Systems), the LAD was overinflated (balloon/artery diameter=1.4, 3 inflations and endothelial denudation rub) with an angioplasty catheter (Intrepid, 7 ATM, Baxter Healthcare Laboratories) in an AR2-guide catheter (Scimed Boston Scientific Corporation). Pigs received aspirin (17 mg/kg BID) and an atherogenic diet (15% lard, 1.5% cholesterol mixed in swine minipig chow, Purina Mills, Inc) throughout the study.

One month following plaque creation, pigs underwent PTCA. Anesthesia, cannulations, and drug administrations were as described above. Plaque locus was determined from initial angiograms (first surgery). The balloon was expanded to achieve a 30% increase in lumen diameter. Following PTCA (three 20-second dilations), angiograms (postPTCA) were taken. External jugular vein access ports were implanted, subcutaneously, caudal to the scapula, to facilitate repeat blood withdrawal.

Pigs received RPR101511A (30 mg/kg) or vehicle by feeding tube twice daily for 28 days, beginning 2 hours before PTCA. A dosing suspension was prepared by mixing RPR101511A (Polytron tissumizer, Tekmar) with vehicle (0.5% methylcellulose [final]) and karo syrup, 50:50).

Twenty-eight days following PTCA, final angiograms (terminal-PTCA) were taken and pigs were euthanized with pentobarbital (78 mg/kg). The heart was removed and pressure perfusion fixed for 3 hours with 10% buffered formalin.

Histological Evaluation

Coronary arteries (LAD, circumflex) were cut into 4 mm sections, embedded in paraffin, and stained with Verhoeff's van Gieson and Alcian Blue PAS (EPL Inc). A portion of each section was deformalized, frozen, sliced, and stained with oil red O for lipid deposition. Four 5- μ m side-by-side views from 5 levels within each section were morphometrically and colorimetrically evaluated with a Nikon microscope and attached to a drawing tube with Sigma Scan software and/or an Olympus Vanox-S linked to ImagePro computer analysis system (I-Cube). The circumference of designated areas, injury index, and the number of internal elastic lamina (IEL) fragments were also quantified. Histomorphometric analyses were performed by 3 scientists blinded to treatment.

Angiographic Analysis

Lumen diameters on angiograms were determined by densitometric analysis (Pharmacia LKB Biotechnology Inc). Eight measurements were made spanning the lesion, and 3 measurements were made along the dye-filled catheter. The location of the MLD, identified on the prePTCA angiogram, was used to obtain lumen diameters at the corresponding locus on post- and terminal-angiograms. The circumflex artery was evaluated as the reference artery in a similar manner. Angiograms were analyzed by 2 researchers blinded to treatment.

Plasma and Pharmacokinetic Analyses

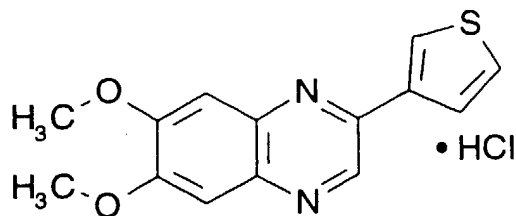
Venous blood samples were obtained during PTCA surgery (day 0) and on days 7, 14, 21, and 27 following angioplasty, 2 hours after the morning dose. Plasma was analyzed for cholesterol by the Hitachi 717 Chemistry Analyzer (Boehringer Mannheim Corp). For quantitation of RPR101511A concentration, plasma was diluted with acetonitrile and analyzed by HPLC fluorescence assay using a LiChrospher RP18 with a linear range of detection of 0.01 to 5 μ g/mL.

Reagents

Cell culture media and human aortic VSMC were purchased from Clonetics. Porcine PDGF-BB and recombinant human PDGF-AA were products of R&D; RPR101511A was synthesized by Medicinal Chemistry, RPR (Collegeville, Pa), according to patented synthesis.¹⁹ Antibodies were purchased from Transduction Laboratories and Genzyme.

Statistics

Results are presented as the mean \pm SEM. Statistical significance was calculated using the paired *t* test for means and the Student's *t* test assuming equal variance. IC₅₀ values were determined from linear regression analysis of the percent inhibition from a minimum of 4 concentrations of RPR101511A.



RPR 101511A

Figure 1. Chemical Structure of RPR101511A.

Results

In Vitro Effects of RPR101511A

RPR101511A (6,7dimethoxy-2-thiophen-3-yl-quinoxaline hydrochloride) (Figure 1), is a potent inhibitor of the human cell-free PDGF β r-TK with an IC_{50} value of 106 nmol/L (Figure 2) and is selective for the PDGF β r-TK compared with other tyrosine kinases (EGFr, CSF-1r) and serine/threonine kinases (PKC, PKA) (Table 1). In a dose-related manner, RPR101511A inhibited in situ PDGF β r-TK autophosphorylation stimulated by PDGF (Figure 3A) and PDGF-dependent mitogenesis in swine coronary SMC (SCSMC), the relevant cell type in our in vivo restenosis model (Figure 3B). IC_{50} values (nmol/L) were 220 ± 120 ($n=3$) and 254 ± 90 ($n=7$) for the 2 activities, respectively. At concentrations slightly higher than those required to inhibit SCSMC mitogenesis, RPR101511A inhibited PDGF β r autophosphorylation, and PDGF-dependent chemotaxis and mitogenesis in human aortic SMC (Table 2). In a 6-day cell growth assay, a single application of RPR101511A retarded PDGF-dependent growth (Table 2). RPR101511A inhibited PDGF-AA-stimulated mitogenesis suggesting that RPR101511A also blocked the PDGF α r-TK (Table 2).

Initial signs of cytotoxicity were not evident until RPR101511A was added at a concentration which exceeded IC_{50} values by 100-fold (ie, 100 μ mol/L), demonstrating that the inhibition of PDGF-dependent activities was not mediated by nonspecific VSMC cytotoxicity.

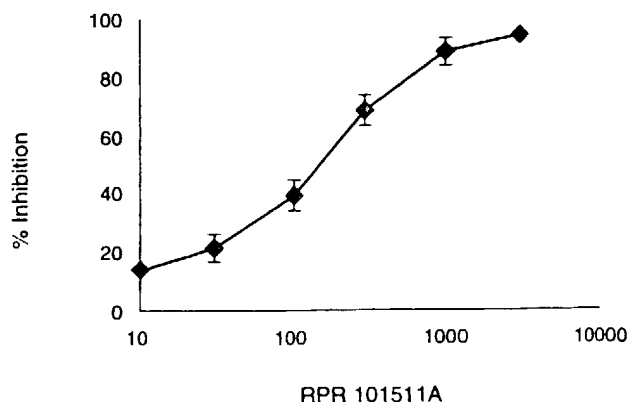


Figure 2. Effect of RPR101511A on cell-free PDGF β r-TK activity. PDGF-stimulated autophosphorylation of the PDGF β r immunoprecipitated from human VSMC was evaluated by an ELISA assay, described in Methods. $n=15$ separate experiments.

TABLE 1. Effect of RPR101511A on Kinase Activity

Kinase	IC_{50} , nmol/L
PDGF β r	106 ± 6 (15)
EGFr	$>50\ 000$ (3)
CSF-1r	$>30\ 000$ (3)
PKC	$>100\ 000$ (3)
PKA	$>100\ 000$ (3)

Kinase activity was measured as described in Methods. IC_{50} values given as mean \pm SEM. Values in parentheses represent number of separate experiments. EGFr indicates epidermal growth factor receptor and CSF-1r, colony-stimulating factor-1 receptor.

Inhibition of Angiographic Restenosis With RPR101511A

To evaluate the effects of a selective PDGF β r-TKI on coronary artery restenosis, RPR101511A was administered BID, beginning 2 hours before PTCA and continuing for 28 days. PrePTCA MLD of the vehicle and RPR101511A-treated pigs were similar (Figure 4). Following successful dilation, the MLD increased significantly and to the same extent in both groups. Twenty-eight days following PTCA and oral treatment with vehicle or RPR101511A, there was a significant decrease in the MLD of vehicle-treated pigs but no angiographic change in the MLD of RPR101511A-treated pigs. Thus, 54% (7 of 13) of the vehicle-treated pigs exhibited $>50\%$ loss of gain compared with 23% (3 of 13) of RPR101511A-treated pigs. This represents a 57% decrease in restenotic rate. As shown by the equivalency of gain in the

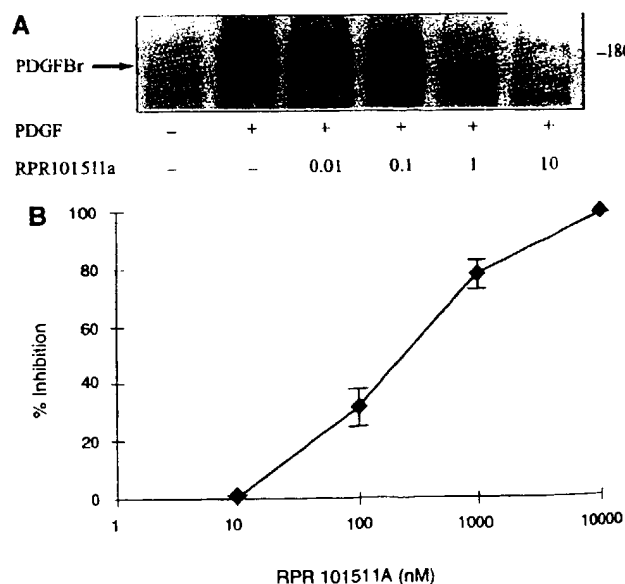


Figure 3. Effect of RPR101511A on inhibition of PDGF-stimulated in situ autophosphorylation and mitogenesis in cultured swine coronary VSMC. A, PDGF β r from lysates (70 μ g protein per lane) of coronary VSMC treated with RPR101511A and PDGF as described in Methods. Molecular weight marker shown to right identifies the PDGF β r at 180 kDa. Confirmation of the receptor was determined by western blotting with anti-PDGF β r antibodies (data not shown). B, Cells were growth-arrested, pretreated with RPR101511A at doses shown, and stimulated with PDGF (10 ng/mL). Proliferation was assayed by 3 H-thymidine incorporation. $n=9$ separate experiments. VSMC indicates vascular smooth muscle cell.

TABLE 2. Effect of RPR101511A on PDGFr-Dependent Activities of Human VSMC

Cellular Activity	IC ₅₀ , nmol/L
In situ autophosphorylation	631±239 (4)
Mitogenesis	
PDGF-BB	605±81 (22)
PDGF-AA	559±89 (7)*
Chemotaxis	492±86 (4)
Cell Growth	4400±1300 (4)

Cellular activities were measured as described in Methods. The concentration of PDGF used for IC₅₀ determination produced 80% of maximal response. IC₅₀ values given as mean±SEM. Values in parentheses represent number separate experiments.

*Cultures stimulated with PDGF-AA.

o groups, by the similar angiographically determined lumen to artery ratio in the 2 groups (Table 3) and by the lack of change in the reference diameter of the 2 groups over time (Table 3), the beneficial effect of RPR101511A on the

TABLE 3. Angiographic Analysis

Parameter	Vehicle	RPR101511A	P
BD/AD*	1.57±0.11	1.47±0.10	0.11
Circumflex artery diameter, mm†			
PostPTCA	1.96±0.05	1.82±0.05	0.11
Termination	1.84±0.05	1.78±0.05	0.12

BD indicates balloon diameter and AD, artery diameter.

*Angiographically determined balloon and artery diameters at site of MLD.

†Locus of diameter measurement for circumflex coronary artery was selected on postPTCA angiogram and compared with corresponding locus on terminal angiogram. Circumflex angiograms were available for 13 vehicle-treated pigs and 10 RPR101511A-treated pigs.

MLD following angioplasty was unlikely attributable to variable degrees of injury or angiographic artifacts.

Inhibition of Histological Intimal Hyperplasia by RPR101511A

Figure 5 shows representative examples of histologically stained arterial lesions from vehicle-treated (left) and RPR101511A-treated (right) pigs. Total neointimal area, defined as the region between the lumen and the IEL, is the complex lesion generated by the initial injury plus the atherogenic diet albeit altered by PTCA compression and the intimal growth which occurred during the 28 days following PTCA. Total intimal area and total intimal/medial area (I/M) ratios of sections displaying the minimal luminal area were significantly reduced in tissues removed from pigs treated with RPR101511A compared with vehicle (Figure 6). The average histomorphometric minimal lumen area, lumen circumference, adventitial area, artery area, and medial area were not significantly different between RPR101511A-treated and control preparations.

Total intimal area and I/M ratios of the entire lesion were reduced with RPR101511A treatment (Table 4). However, statistical significance was not achieved. Lumen area, medial area, and artery areas were similar in the 2 groups (data not shown). The extent of injury as estimated by the injury index and the number of IEL fragments throughout the lesion were comparable in vehicle and RPR101511A-treated pigs suggesting that the applied injury was similar in the 2 groups. This is consistent with the angiographic data. In addition, the EEL and IEL areas of the reference circumflex artery untouched by the balloon catheter were comparable in the 2 groups (data not shown).

Vasoreactivity

Maximal isometric tension induced by three vasoconstrictor agonists in porcine coronary artery rings and the effect of RPR101511A on this vasoreactivity are given in Table 5. RPR101511A significantly inhibited PDGF and angiotensin-mediated vasoconstriction but had no effect on serotonin-induced vasoconstriction.

RPR101511A Plasma Concentration

Pharmacokinetic studies conducted with RPR101511A in the Yucatan minipig demonstrate a T_{max} of 2 hours and a T_{1/2} of 8 hours following oral administration. On the basis of these

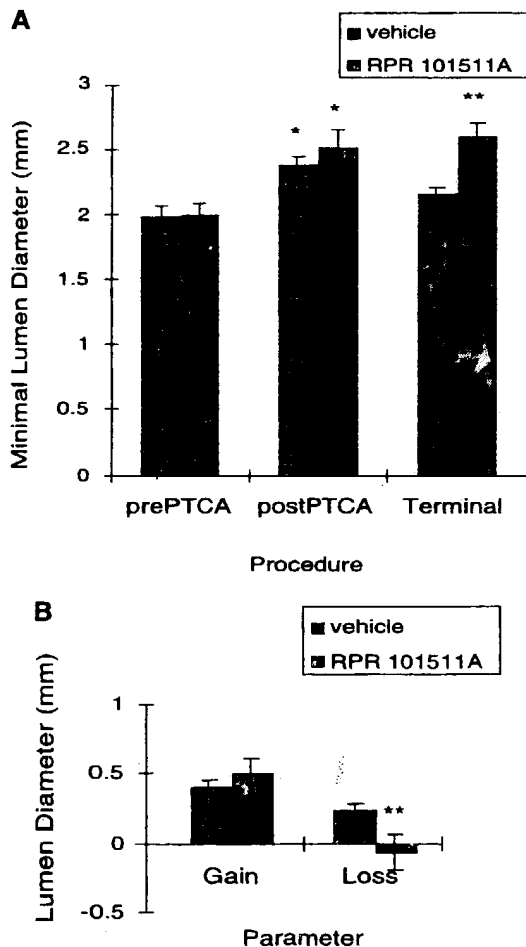


Figure 4. Effect of RPR101511A on angiographically determined MLD of the swine LAD artery. A, MLD from prePTCA, postPTCA, and terminal angiograms of control (n=13) and RPR101511A-treated pigs (n=13) were expressed as mean±SEM. * *P*<0.05 compared with prePTCA within the same group. ***P*<0.05 compared with vehicle. B, Gain=change in MLD produced by PTCA and loss=change in MLD from postPTCA to termination. ***P*<0.05 compared with vehicle. LAD indicates left anterior descending; MLD, mean lumen diameter.

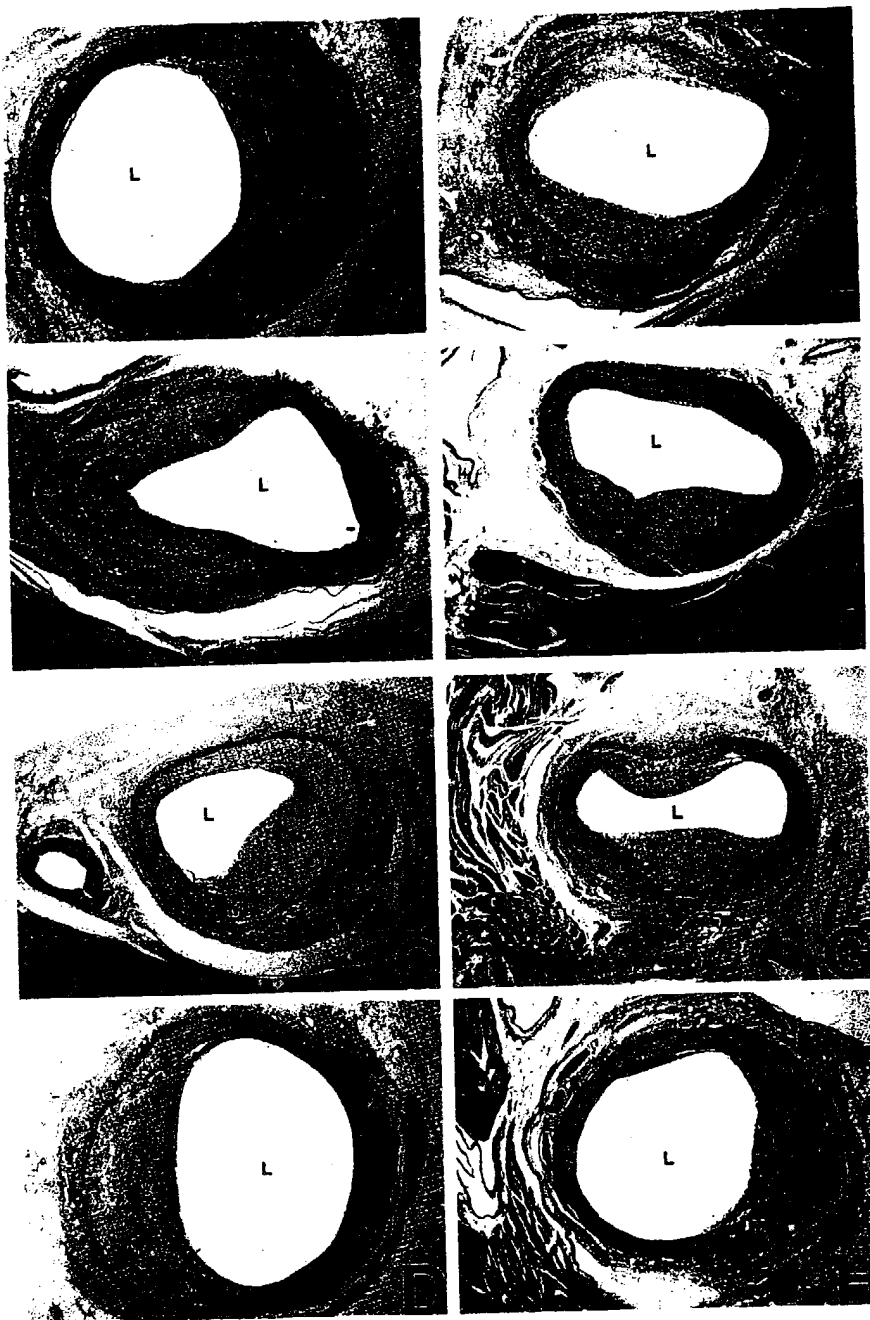


Figure 5. Effect of RPR101511A on histology 28 days after PTCA. Representative views of LAD from vehicle-treated pigs (A-D) and RPR101511A-treated pigs (E-H). LAD was stained with Verhoeff's van Gieson stain. L indicates lumen; I, intima; and M, media. Original magnification $\times 40$.

results, plasma samples were collected 2 hours postdosing every 7 days throughout the study. The mean plasma concentration in RPR101511A-treated pigs ranged from 200 to 400 ng/mL (0.65 to 1.29 $\mu\text{mol/L}$) during the study (Figure 7), concentrations in excess of those used to inhibit PDGF-dependent mitogenesis of SCSMC in vitro (Figure 3).

Plasma Cholesterol

Because pigs were fed an atherogenic diet throughout the study, plasma cholesterol increased from time of PTCA surgery to termination. The increase in the control group was not significantly different from the RPR101511A-treated group (mean change in total cholesterol from day of PTCA to termination, control, 63 ± 58 mg/dL; RPR101511A-treated, 110 ± 86 mg/dL). Additionally, the relative density of

oil red O staining for lipid (vehicle:RPR101511A, mean \pm SEM) in the artery, (7781 ± 990 ; 6988 ± 764) plaque (9167 ± 1166 ; 8213 ± 1004), and the section with the MLD (9841 ± 1864 ; 8704 ± 1298) was similar in the 2 groups.

Discussion

The oral efficacy of the selective PDGFr-TKI, RPR101511A, was evaluated in a porcine model of coronary artery restenosis following PTCA. This model shares similarities with human restenosis in several ways: PTCA was performed on a preestablished coronary lesion in the presence of elevated plasma cholesterol, surgical protocols were similar to those used clinically, lumen diameters were measured before and after PTCA and again 1 month later by angiography (Figure 4) and at termination, the restenotic lesion contained smooth

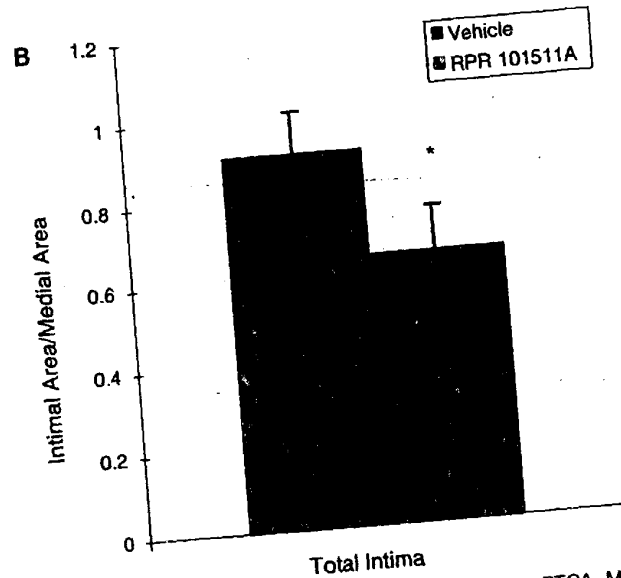
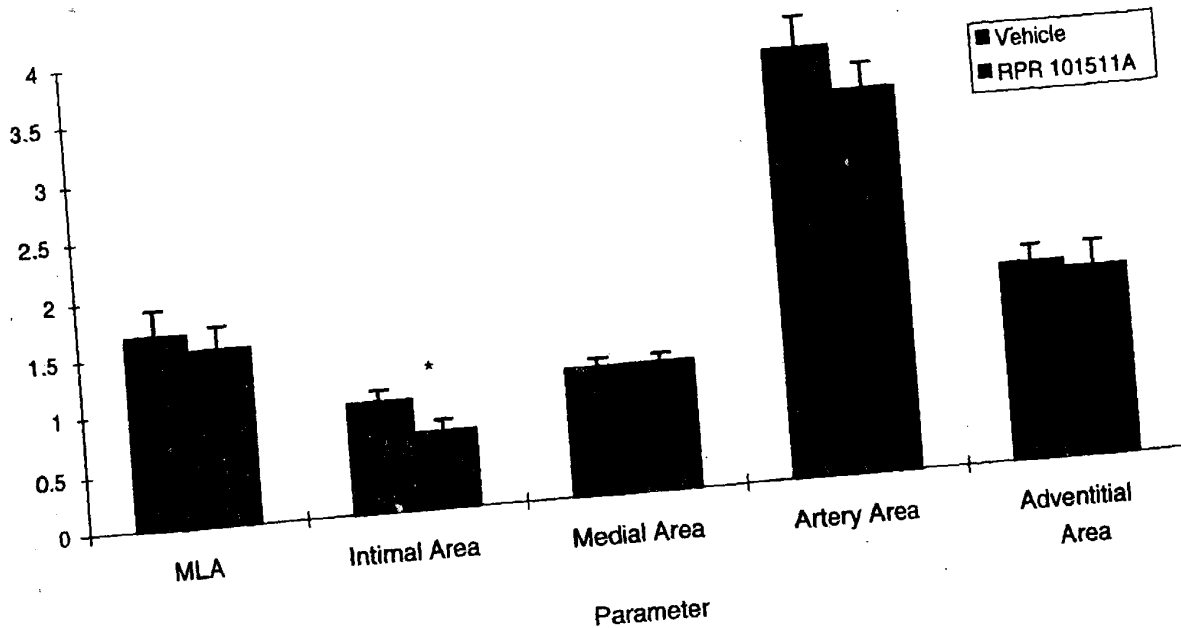


Figure 6. Effect of RPR101511A on histomorphometrics of arteries removed 28 days after PTCA. Measurements were obtained from section with minimal lumen area (MLA). * $P < 0.05$.

muscle cells, matrix, and lipids (Figure 5) comparable to human restenotic lesions.^{20,21} This model differs significantly from previously described models in which naïve coronary arteries of normocholesterolemic pigs were subjected to a single catheter-induced injury.²²⁻²⁴ RPR101511A is a novel, low molecular weight inhibitor of PDGF- α TK autophosphorylation (Figures 2 and 3) and an inhibitor of multiple PDGF-dependent activities of SMC (Figure 3B, Table 2). Although PDGF has long been implicated in the development of restenosis,¹ the concordant expression of ligands and the PDGF β r in human restenotic lesions has only recently been demonstrated^{12,13}; it reinforces the role of PDGF as a critical player in the restenotic reparative process. In this study, RPR101511A was used as a prototype selective inhibitor of the PDGF- α TK to evaluate the role of

PDGF-dependent responses in a porcine model of coronary restenosis. Our data demonstrate that oral administration of RPR101511A prevented angiographically defined restenosis (Figure 4). Pigs treated with RPR101511A exhibited no angiographic loss of gain following PTCA whereas vehicle-treated controls exhibited significant loss of gain (50%). Because both groups received the same degree of angiographic dilation (Table 3, Figure 4), the effect of RPR101511A was unlikely a consequence of differences in applied injury.

Morphometrically, the total amount (ie, resulting from both the initial injury and restenosis) of intimal hyperplasia in coronaries of pigs receiving RPR101511A was significantly smaller (30%) than in vehicle-treated pigs (Figure 6). Because we chose to evaluate the effect of RPR101511A on PTCA-induced restenosis, ie, the vascular response in the

TABLE 4. Morphometric Parameters Averaged Across Lesion

Histological Region/Parameter	Treatment		P
	Vehicle (n=13)	RPR101511A (n=13)	
Total intimal area, mm ²	0.82±0.06	0.64±0.08	0.053
Total I/M	0.80±0.05	0.66±0.08	0.08
Injury Index	0.25±0.01	0.22±0.03	NS
IEL fragments (average)	1.00±0.20	2.00±0.23	NS
IEL fragments (total)	6.00±0.81	7.00±0.92	NS

Morphometric measurements were averaged from all sections within the lesion. Intimal area indicates region from lumen to IEL; I/M, intimal area to medial area; injury index, distance between IEL fragments/IEL circumference. IEL fragments measured in 4 contiguous sections. Values given as mean±SEM.

presence of a preexisting lesion, morphometric quantitation of the intimal growth post PTCA was not possible and hence only total intimal lesion data are presented. Thus, one explanation for the modest inhibitory effect of RPR101511A on intimal hyperplasia may relate to our inability to measure selectively PTCA-induced restenotic intimal growth. Although attempts were made to detect restenotic growth by differential staining (eg, proteoaminoglycan sulfation with Alcian blue PAS),²⁵ this will be more meaningfully accomplished by the use of intravascular ultrasound and will be included in subsequent reports.

From in vitro characterization of RPR101511A as a potent inhibitor of PDGF-dependent migration and proliferation in VSMC (Table 2, Figure 3B) and from plasma levels of RPR101511A (Figure 7) achieved in this study which exceeded the in vitro IC₅₀ values (Table 2), it is reasonable to propose that inhibition of restenosis may occur by inhibition of cellular activities initiated by PDGFr activation. Although RPR101511A is without effect on EGFr-TK, CSF-1r-TK, and PKC and PKA (Table 1), an effect on the TK receptors for insulin-like growth factor-1 and fibroblast growth factor, growth factors also implicated in restenosis,¹ cannot be excluded. In addition, it is possible that RPR101511A exerted a favorable vasodilatory effect on coronary arteries by inhibition of vasoconstriction induced by PDGF or angiotensin II (Table 5). However, the role of vasoconstrictors in restenosis is unknown. RPR101511A reduced restenosis independent of an effect on plasma cholesterol and arterial wall accumulation of lipid.

TABLE 5. Effect of RPR101511A on Vasoreactivity to Select Agonists in Porcine Coronary Artery Rings

Agonist	Maximal Isometric Tension, g	
	Control	RPR101511A
Serotonin	7.0±1.3 (4)	8.1±1.5 (7)
Angiotensin	15.1±1.0 (4)	8.1±2.6 (7)*
PDGF	5.4±1.8 (6)	0.4±0.2 (4)*

Porcine coronary artery rings were preincubated with RPR101511A (1 µmol/L) and stimulated with increasing concentrations of select vasoconstrictors. Values given as mean±SEM. Number of separate experiments given in parentheses.

*P<0.05.

Plasma Concentration of RPR 101511A

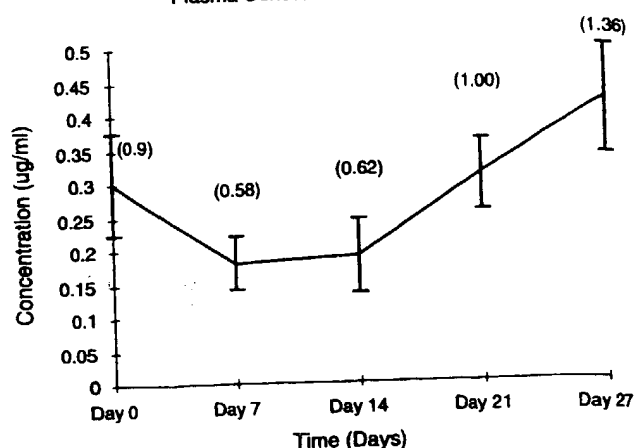


Figure 7. Plasma concentration of RPR101511A during treatment. RPR101511A was not detected in plasma (<0.010 µg/mL) from vehicle-treated pigs. Numbers above line represent µmol/L equivalent values.

For results to be meaningful, according to the double injury model, the applied injury both during plaque creation and at PTCA must be the same in experimental and control animals. Because intravascular ultrasound was not used, the extent of injury can only be determined indirectly. This represents a limitation to this study. As with animal stenosis models, an injury index is generated and as shown here, this was similar in control and treatment groups (Table 4). Another determinant of injury, one which would tend to accumulate throughout the study, is the number of IEL fragments. Our data show that the number of breaks in 4 contiguous lesion sections were the same in both groups (Table 4). Angiographic data provide balloon/artery ratios (Table 3) which indicate that equal degrees of injury were applied to the LAD of both groups.

Currently, porcine models of (re)stenosis are considered to be the best approach for evaluating potential new therapies to modulate restenosis and to predict their success in man. In pig stenosis models, angiopeptin (a somatostatin analogue)²² and probucol, an antioxidant,²³ have shown variable degrees of inhibition of intimal thickness, I/M ratio, and intimal area. They have similarly demonstrated some degree of clinical success in inhibition of restenosis.²⁶⁻²⁸ These results suggest that porcine models of (re)stenosis may be more predictable of the clinical outcomes using new treatment modalities than previous rat and rabbit models have been. Nonetheless, it is clear that there remains a void in the therapy for restenosis.

Acknowledgments

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PDGF β Receptor Blockade Inhibits Intimal Hyperplasia in the Baboon

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Background—We have evaluated the use of a mouse/human chimeric anti-platelet-derived growth factor- β receptor antibody in combination with heparin to inhibit intimal hyperplasia in the saphenous artery of the baboon after balloon angioplasty.

Methods and Results—The study evaluated lesion development in sequential injuries made 28 days apart. Each animal received control treatment after the first injury and antibody/heparin therapy after the second injury to the contralateral artery. The antibody was administered by bolus intravenous injections (10 mg/kg) on study days 1, 4, 8, 15, and 22 and heparin coadministered by continuous intravenous infusion at a dose of 0.13 mg/kg per hour. Morphometric analysis of tissue sections showed a 53% decrease in intimal area after antibody/heparin treatment ($P=0.005$), corresponding to a 40% decrease in the intima-to-media ratio ($P=0.005$). Smooth muscle cell proliferation in the injured wall, measured at both 4 and 29 days after balloon injury, were similar in the control and antibody/heparin-treated animals.

Conclusions—These data suggest that platelet-derived growth factor plays a key role in the development of intimal lesions at sites of acute vascular injury in the nonhuman primate. (*Circulation*. 1999;99:564-569.)

Key Words: restenosis ■ angioplasty ■ balloon ■ receptors ■ antibodies ■ platelet-derived factors

Numerous studies have addressed the potential role of platelet-derived growth factor (PDGF) on the development of intimal hyperplasia at sites of acute vascular injury.¹⁻⁴ With the use of an antiplatelet antibody to make rats thrombocytopenic, Fingerle et al² demonstrated that platelets were required for the development of intimal hyperplasia in the rat carotid artery after balloon injury. Subsequently, Ferns et al³ demonstrated that administration of a goat polyclonal antibody that reacts with all 3 dimeric forms of PDGF (AA, AB, BB) was able to inhibit intimal hyperplasia in the rat carotid artery after balloon injury but had no effect on smooth muscle cell (SMC) proliferation. Jawien et al⁴ showed that addition of PDGF-BB to a rat after gentle injury to the carotid artery led to an increase in lesion size, probably by stimulating SMC migration. Recently Sirois et al¹ used an antisense approach to address the role of the PDGF- β receptor (PDGFR- β) in lesion development in the rat balloon injury model. They observed that blockade of PDGFR- β production through local administration of a PDGFR- β antisense construct directly to the injured carotid artery also led to a marked decrease in the extent of lesion development.

PDGF ligand and PDGF receptors have been detected in developing vascular lesions in humans and baboons,⁵⁻⁸ but

their function has not been defined in part because specific inhibitors have not been available. The development of a specific neutralizing antibody to the PDGF- β receptor now makes these studies possible.⁹ Both the α and β receptors, when activated, can mediate a mitogenic signal. In contrast, only activation of the β receptor appears to be capable of eliciting a chemotactic response in vascular SMCs. Blockade of the β receptor with a monoclonal antibody, followed by the addition of either PDGF-BB or PDGF-AA, activates the α receptor and leads to inhibition of SMC migration stimulated by fibronectin.⁹

Unfractionated and low-molecular-weight heparin are potent inhibitors of intimal hyperplasia in the rat balloon injury model.¹⁰ When low-molecular-weight heparin was used in a baboon balloon injury model, no inhibitory activity was observed on intimal lesion development, even at a dose of 0.6 mg/kg per hour over a 28-day period.¹¹ However, we have recently shown that heparin, given together with a blocking antibody to the PDGF- β receptor, inhibits SMC migration from fresh aortic explants obtained from the baboon (R. Kenagy, C. Hart, A. Clowes, unpublished observation, 1997).

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The observation that heparin treatment alone has no inhibitory activity but in association with anti-PDGF receptor antibodies blocked SMC migration provided the basis for the *in vivo* experiments described in this report.

Methods

Source of Antibodies

A murine monoclonal antibody, 163.3.1.1, was generated against the human PDGF- β receptor.^{9,12} A mouse/human chimeric version of this antibody, containing the variable domains for the heavy and light chains of the parent murine antibody, and the constant domains of human IgG₁ and human κ for the heavy and light chains, respectively, was subsequently generated with the use of previously described methods.^{13,14} The antibody was formulated at 10 mg/mL in 50 mmol/L sodium acetate, 125 mmol/L NaCl, pH 5.0.

Source of Heparin

Porcine heparin, grade II, 157 U/mg (Sigma Chemical Co), was formulated into a 0.9% sterile saline solution. Two Alzet osmotic pumps (Alza Corp) were placed subcutaneously into each animal, and the heparin (0.13 mg/kg per hour) or vehicle control was delivered through a catheter inserted into the femoral vein.

In Vivo Study Protocols

Study 1 evaluated the ability of the chimeric antibody, in combination with heparin, to inhibit intimal hyperplasia in the saphenous artery of baboons, measured 29 days after balloon pullback injury. The study was divided into a control phase and an antibody treatment phase. For each study phase, a 10-cm section of 1 saphenous artery was injured with a 2F Fogarty embolectomy catheter as previously described.¹¹ In the first phase, the animals received antibody vehicle control injections on study days 1, 4, 8, 15, and 22 after balloon injury and continuous intravenous infusion into the femoral vein of saline control. Study day 1 was the day of the surgery. On day 28 after the injury, the animals received 3 injections of bromodeoxyuridine (BrdU) (300 mg/dose IM) at 17 hours, 9 hours, and 1 hour before tissue collection. At study day 29, the injured artery was surgically removed and processed for tissue analysis. After removal of the first artery, the contralateral saphenous artery was immediately balloon-injured. Anti-PDGF receptor antibody was injected intravenously on study days 1, 4, 8, 15, and 22 at the dose of 10 mg/kg and heparin infused intravenously at a rate of 0.13 mg/kg per hour. Plasma and serum samples were obtained immediately before each subsequent antibody injection. On day 28 after the second injury, the animals received 3 injections of BrdU as previously described. At study day 29, the injured artery was surgically removed and processed for tissue analysis. Fifteen male animals (*Papio cynocephalus*), 7 to 12 kg in weight, were entered into the study. Ketamine (10 mg/kg) was used as a preanesthetic, and the animals were maintained on isoflurane during surgery. The operations were performed at Biosupport (Redmond, Wash), an AALAC-approved animal facility. The protocol was approved by the Animal Use and Care Committee at Biosupport, and all procedures followed Good Laboratory Practices as defined in current FDA, 21 CFR Part 58.

Study 2 used a sequential injury model to evaluate the ability of the chimeric antibody, in combination with heparin, to inhibit SMC replication at 4 days after balloon injury to the saphenous artery. After the first injury, the animals received a single bolus intravenous injection of antibody vehicle control (day 1) and infusion of saline through Alzet pumps into the femoral vein. On day 3, the animals received 3 injections of BrdU before tissue collection. On day 4, blood was drawn for serum collection, and the injured artery was surgically excised. The harvested artery was cut into multiple sections and processed for tissue analysis. Three pieces were placed into 10% formalin fixation for BrdU staining and morphometric analysis. The remaining segments were microdissected to separate the adventitia from the media, and the 2-vessel wall components were frozen separately for subsequent analysis. Adjacent noninjured

saphenous artery and sections of carotid and axillary artery were also collected and similarly processed for analysis.

On day 4, before injuring the contralateral saphenous artery but after removal of the first injured artery, the animals received a single bolus intravenous injection of the chimeric anti-PDGF- β receptor antibody (10 mg/kg). The femoral vein was cannulated and heparin infused through Alzet pumps. At day 3 after the second injury, the animals received 3 injections of BrdU. On day 4, serum was collected and the second injured saphenous artery, along with noninjured saphenous, carotid, and axillary arteries, was excised and processed as described above. Five animals (*Papio cynocephalus*), weighing between 8 and 10 kg, were entered into study 2. The animals were preanesthetized with ketamine (10 mg/kg) and maintained under general isoflurane during surgery. All surgical procedures were done at the University of Washington Primate Center under guidelines approved by the University of Washington Animal Use and Care Committee.

ELISA to Measure Chimeric Anti-PDGF- β Receptor Antibody Levels

Ninety-six-well microtiter plates were coated with IgG/PDGF- β fusion protein¹² at 200 ng/mL diluted in coating buffer (0.1 mol/L Na₂HCO₃, pH 9.6). The plates were incubated overnight at 4°C, washed with ELISA C buffer (PBS, 0.05% Tween 20), then blocked with ELISA B buffer (PBS, 0.1% BSA, 0.05% Tween-20). Serum sample dilutions were made in ELISA B buffer. Standard curves were generated with purified chimeric antibody diluted into control baboon serum. Samples were added to duplicate wells and incubated at 37°C for 2 hours. The plates were washed to remove unbound antibody, and a 1:600 dilution of goat anti-human IgG₁ antibody conjugated to horseradish peroxidase (Zymed) was added to the wells for 1 hour at 37°C. The wells were washed with ELISA C buffer, then incubated with OPD substrate solution (12.5 mL 0.1 mol/L Na citrate, pH 5.0, 5 mg *o*-phenylenediamine, 5 μ L 30% H₂O₂). The reaction was stopped by the addition of 1N H₂SO₄ and the plates read at absorbance 490 nm in a Dynatech ELISA plate reader (Molecular Devices).

Activated Partial Thromboplastin Time Analysis to Monitor Levels of Circulating Heparin

Plasma was collected at various times after implantation of the Alzet osmotic pumps, and the heparin levels were monitored by activated partial thromboplastin time (APTT) analysis with the HEPTEST Assay (Product # 803, American Diagnostica) in an MLA Electra 800 clotting machine.

Morphometric Analysis and BrdU Staining of Arterial Tissue Sections

For study 1, the excised ballooned arteries were perfusion-fixed at 100 mm Hg *ex vivo* with 10% formalin. Pieces of artery were embedded in paraffin, and 5- μ m sections were cut from each tissue block for morphometric analysis as described by Geary et al.¹¹ Tissue sections from study 2 fixed in formalin were similarly embedded in paraffin, and sections were cut from each block for analysis. Sections from both study 1 and study 2 were stained with a monoclonal antibody against BrdU (Boehringer Mannheim Corp) with an immunoperoxidase method as previously described¹¹ to measure the level of cell replication.

Extraction of Baboon Aortic Tissues

Frozen baboon arterial segments were placed in a glass tissue homogenizer and 1 mL of TNEN buffer (20 mmol/L Tris base, pH 8.0, 100 mmol/L NaCl, 5 mmol/L EDTA, 0.5% nonidet-40, 1 mmol/L PMSF, 50U/mL aprotinin) added per 0.25 g of tissue. The tissues were homogenized on ice over a 10-minute period, and the homogenate was transferred to an Eppendorf tube and spun for 5 minutes in a microfuge. The supernatant was harvested and frozen at -80°C until assayed.

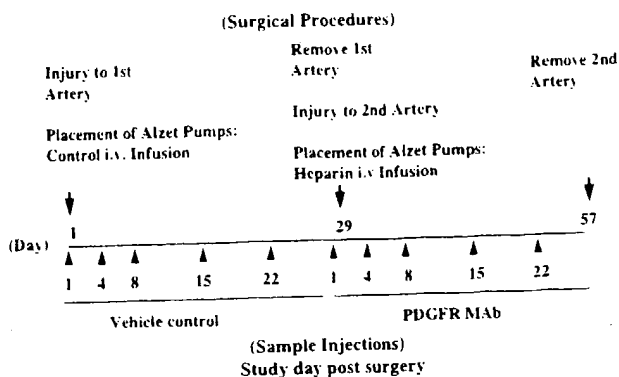


Figure 1. Experimental protocol for study 1. Chimeric anti-PDGFR- β receptor antibody was evaluated for its ability to inhibit intimal hyperplasia in balloon-injured baboon saphenous artery. Sequential balloon injury was performed 28 days apart to the saphenous arteries. Control injections and infusions were given after first injury; anti-PDGFR- β receptor antibody injections and heparin infusions were given after second injury.

Immunostaining to Detect Chimeric Antibody in Tissues

Histological cross sections of paraffin-embedded tissue were mounted on positively charged microscope slides (Superfrost Plus, Curtin Matheson). Slides were deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase was blocked with 3% H_2O_2 , followed by enzymatic pretreatment with 0.1% trypsin in 0.05 mol/L Tris buffer (pH 7.6) for 30 minutes at 37°C. A peroxidase-conjugated mouse monoclonal antibody to human IgG₁ (Zymed Laboratories, Inc) was applied to the slides overnight at 4°C at a dilution of 1:10 in 1% BSA in PBS buffer (pH 7.3). Bound antibody complexes were visualized with the use of diaminobenzidine as the chromogen.

Results

Study Protocol Design

The addition of anti-PDGFR- β antibody 163.3.1.1 to baboon SMCs in culture causes a dose-dependent decrease in [3H]thymidine incorporation, with 25 μ g/mL of antibody able to inhibit >50% of the mitogenic activity in baboon serum (data not shown). The addition of 10 μ g/mL of heparin alone to these cells suppresses mitogenic activity by 31%. When the antibody is added to the cells in combination with heparin, a cooperative inhibitory effect is observed, such that a dose of 1 μ g/mL of the antibody, in the presence of 10 μ g/mL of heparin, has inhibitory activity equal to 25 μ g/mL of antibody alone (data not shown). These results suggest that coadministration of heparin with the anti-PDGFR- β receptor antibody would enhance the ability of the antibody to inhibit smooth muscle cell activation in the in vivo studies.

The in vivo studies described in this report were designed to approximate a clinical study for the evaluation of PDGFR- β blockade by antibody 163.3.1.1. Because of the cooperative effect between heparin and the anti-PDGFR- β antibody to inhibit SMC replication, we designed the study to deliver both antibody and low-dose heparin. A chart describing study 1 is presented in Figure 1.

To minimize animal-to-animal variation in lesion development, we used sequential injuries within each animal for evaluation of the test compounds. A concern before the initiation of the study was what effect the excision of the first

TABLE 1. Morphometry of Balloon-Injured Saphenous Arteries at 29 Days

	Intimal Area, mm ²	Medial Area, mm ²	M Ratio
Control*	0.081 \pm 0.047	0.762 \pm 0.157	0.098 \pm 0.045
Anti-PDGFR*	0.038 \pm 0.012	0.637 \pm 0.105	0.059 \pm 0.018
	53% Decrease	16% Decrease	40% Decrease
	P=0.005	P=0.003	P=0.005

Data are mean \pm SD (n=12). Statistical analysis was done with paired Student *t* test. One-tailed test was done for intimal area and M ratios and 2-tailed test for medial area.

injured saphenous artery would have on lesion development in the contralateral injured artery. To address this question, a preliminary study was run in which 2 control animals received sequential balloon injuries to their saphenous arteries 29 days apart. The arteries were removed, the vessels perfusion-fixed ex vivo, and the tissues processed for morphometric analysis. Evaluation of intima/media ratios, as a measurement of lesion development, from cross sections of the paired arteries showed that there was no significant difference between the first and second injured arteries (data not shown).

Circulating Levels of Chimeric Antibody

Circulating chimeric antibody levels for study 1 were determined for serum samples collected on study days 8, 15, 22, and 29, immediately before the next antibody injection. The average circulating antibody level was 60.9 \pm 8.7 μ g/mL (mean \pm SD) at day 8, with mean antibody levels decreasing to 36.1 \pm 26.5, 15.4 \pm 23.3, and 11.5 \pm 23.7 μ g/mL on days 15, 22, and 28, respectively. The decrease in circulating antibody level with time appeared to be directly related to the extent of the immune response generated by the baboon toward the chimeric antibody (data not shown).

APTT Analysis

The analysis of plasma samples from study 1 showed a background clot time of 24.3 \pm 2.2 seconds (mean \pm SD). Heparin infusion, 0.13 mg/kg per hour, caused an increase in APTT that ranged from 2- to 4-fold and was maintained throughout the length of the study. Specific APTT times were 55.8 \pm 13.4, 46.9 \pm 17.2, 42.6 \pm 15.9, and 53.8 \pm 18.5 seconds (mean \pm SD) on study days 8, 15, 22, and 29, respectively. No bleeding complications were observed in the animals. The circulating levels of heparin ranged from 0.25 to 0.5 U/mL, corresponding to a level of 2 to 3 μ g/mL of heparin.

Morphometric Analysis of Tissue Sections

Tissue sections from study 1 were obtained from multiple blocks of each test artery. Absolute intimal and medial areas were determined for each tissue section, and the data for all the blocks were averaged to give mean values for each animal. The data from 3 animals were eliminated from analysis because of the presence of occluding thrombi at the site of balloon angioplasty in either one or both arteries. A summary of the intima (I) and medial (M) areas and I/M ratios for the 12 remaining animals are given in Table 1. The

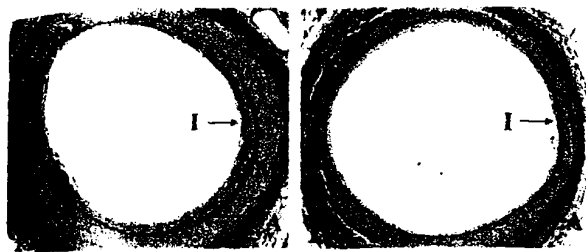


Figure 2. Photomicrographs of cross sections of balloon-injured arteries: cross sections of saphenous arteries 29 days after balloon injury (study 1). Sections 5 μ m thick were cut and stained with Verhoeff-van Gieson's stain. A, Vehicle control-treated artery; B, anti-PDGF- β receptor antibody-treated artery. I indicates intima; M, media; A, adventitia. Bar=100 μ m.

intimal areas were decreased significantly (53%) in the antibody-treated arteries ($P=0.005$). Analysis of the I/M ratios, to minimize differences in the absolute size of the arteries, also demonstrated a highly significant decrease (40%) in the antibody-treated animals ($P=0.005$). A photomicrograph of representative cross sections of injured arteries is shown in Figure 2.

Cell Proliferation in Injured Vessels

Intimal and medial SMC proliferation, as determined by BrdU labeling (Tables 2 and 3), was not significantly different between the antibody-treated and vehicle control-treated arteries at either 4 or 29 days after injury. However, the total nuclear number was significantly decreased ($P=0.007$) in the intima of the antibody-treated arteries at 29 days.

Detection of Chimeric Antibody 163.3.1 in the Vessel Wall

High levels of the chimeric antibody were detected in the artery wall extracts as determined by ELISA (Figure 3). The level of antibody in the medial portion of the injured saphenous artery was ≈ 8 -fold higher than the level detected in the noninjured artery (952 ng/mg vs 124 ng of antibody/mg of total protein, respectively). The antibody level in the adventitia was also increased in the injured artery compared with the noninjured artery.

Frozen sections obtained from the saphenous artery of the antibody-treated animals were stained to localize the chimeric

antibody in the artery wall. Strong staining for the chimeric antibody was observed in the media of the injured artery segments, whereas a lower level of staining was detected in the adventitia (Figure 4). No staining was detected in adjacent noninjured tissue. The elevated levels of antibody in the adventitia measured by ELISA (Figure 3), as compared with the lower levels detected by immunostaining, probably are due to the presence of trapped blood in the vasa vasorum, which would contain high antibody levels and would be included in the tissue extracts. Processing of the frozen tissue sections for immunostaining would eliminate trapped blood.

Discussion

The studies reported in this article are unique in that it is one of the first times that inhibition of intimal hyperplasia has been demonstrated in a nonhuman primate model of vascular injury. This is a key point because the bulk of restenosis studies have been done in lower mammals including rats, rabbits, and pigs.¹⁵⁻¹⁸ Although many compounds have proven effective in these models to inhibit intimal hyperplasia, all of those further tested in humans have failed to maintain their efficacy.^{15,16} The published observations that heparin is an effective inhibitor of intimal hyperplasia in the rat¹⁹ but having no effect in the baboon¹¹ further supports the concept that primates may respond differently to acute vascular injuries. Although it has been demonstrated that PDGF- β receptor blockade by either antisense or a selective tyrosine kinase inhibitor has been effective in blocking intimal hyperplasia in the rat and pig,^{1,20} it has not been clear that such a response would occur in a primate.

We believe that the baboon response most closely approximates the response that one will see in humans. Although the baboon model we used is not one of preexisting atherosclerotic injury, it is a model in which significant intimal hyperplasia occurs. From a clinical perspective, this most closely approximates the response seen after the placement of vascular stents. Restenosis occurring because of stent placement in humans is the result of true intimal hyperplasia in the absence of vessel wall remodeling associated with vasoconstriction. With the increased use of stents and the continued problem with in-stent restenosis, a specific inhibitor of intimal hyperplasia should have clinical utility. Our data suggest that an anti-PDGF- β receptor antibody may be such a drug.

The decrease in intimal hyperplasia obtained with PDGF- β receptor blockade could be due to a variety of factors. The most likely explanation is inhibition of migration of SMCs from the media to the intima rather than inhibition of SMC replication. This is supported by the decrease in intimal cell number measured in the antibody-treated arteries (Table 2), with no corresponding decrease in replication rate. A similar finding was made by Ferns et al,³ who used an anti-PDGF ligand antibody in a rat balloon injury model. This conclusion is further supported by the observation that systemic administration of PDGF-BB after injury to the rat carotid artery preferentially stimulates cell migration and not cell replication.⁴

When circulating levels of anti-PDGF- β receptor antibody were compared with intimal lesion size measured at 29 days,

TABLE 2. Total Cell Number and BrdU Labeling in Saphenous Artery 28 Days After Balloon Injury

	Placebo		Antibody	
	Cell No.*	% BrdU†	Cell No.*	% BrdU†
Intima	356 \pm 121	5.13 \pm 2.66	232 \pm 50‡	7.08 \pm 4.48
Media	1878 \pm 296	0.27 \pm 0.23	1822 \pm 296	0.24 \pm 0.07

*Data are expressed as total cells per cross-sectional area. Four sections were analyzed for each artery and mean values for all arteries determined. Data are mean \pm SD (n=12).

†Data are presented as percentage of cells replicating in balloon-injured tissue, mean \pm SD, as measured by BrdU incorporation. No significant differences were observed between placebo and antibody-treated arteries in either intima or media.

‡Intimal cell number was significantly lower in antibody-treated arteries ($P=0.007$). Data were analyzed with 2-tailed *t* test of paired data.

TABLE 3. Total Cell Number and BrdU Labeling in Saphenous Artery 4 Days After Balloon Injury

	Placebo			Antibody		
	Injured		Noninjured, % BrdU	Injured		Noninjured, % BrdU
	Cell No.*	% BrdU†		Cell No.	% BrdU	
Intima	72.08 \pm 49	19.2 \pm 10.2	0.0 \pm 0.0	38.8 \pm 18.6	28.2 \pm 13.4	0.4 \pm 0.4
Media	1585.2 \pm 377	4.0 \pm 2.3	0.07 \pm 0.04	1333 \pm 328	10.8 \pm 12.2	0.02 \pm 0.02

*Data are expressed as total cells per cross-sectional area. Four sections were analyzed for each artery and mean values for all arteries determined. Data are mean \pm SD (n=5).

†% BrdU-positive cells was determined by dividing total number of cells present in cross sections by number of cells staining positive for BrdU incorporation.

no correlation was observed (data not shown). This was true for all of the serum collection time points, suggesting that the level of circulating antibody present in the early stages of the study was sufficient to inhibit neointimal lesion formation measured at 29 days. Therefore, a shorter period of antibody administration than the 4 weeks used in this study might be equally effective. From the immunostaining studies, it is clear that the balloon injury provided for free movement of the anti-PDGFR antibody out of the blood and into the artery wall, when compared with noninjured artery sections (Figure 4). This allows the antibody to localize in the artery wall, thereby providing for sustained high concentrations of antibody at the site of the developing intimal lesion.

Although inhibition of PDGF- β receptor for 2 to 4 weeks is sufficient to give inhibition of lesion development at 29 days, it is not known if this inhibition persists at later times. The finding that a 12-hour administration of antibody 7E3 in humans after angioplasty therapy leads to a decrease in the need for repeat revascularization over a 1-year time period²¹ suggests that blockade of initiating events after acute vascular injury may have prolonged benefits regarding changes in vessel wall architecture.

When the size of the lesion at 29 days in the control artery was correlated with the difference in lesion size between the

control and treated arteries (control intima minus treated intima) within each animal, a significant correlation was observed ($r^2=0.908$) (Figure 5). These results suggest that there may be a subgroup of hyperresponders within the baboon population, and it is those animals with the largest lesions in the control arm of the study that showed the biggest benefit from the antibody treatment. If this is the case, then it would be similar to the response seen in humans, in whom only 40% of the individuals undergoing balloon angioplasty exhibit restenosis.

One question that remains to be addressed from these studies is the contribution of heparin to the decrease in intimal thickening. In a previous study conducted in baboons, heparin alone at a 4-fold higher dose had no effect on intimal thickening.¹¹ Because of this finding, we believe that the anti-PDGFR- β receptor antibody—not the heparin—was responsible for the inhibition observed. The lack of efficacy by heparin to inhibit vascular restenosis has similarly been observed in human clinical trials.^{22,23} Although our cell culture studies showed a cooperative effect between the antibody and heparin to inhibit smooth muscle cell proliferation, we believe that the presence of heparin only allowed us to use lower doses of antibody. In data not presented in this article, we have determined that heparin has no direct effect on inhibiting the binding of PDGF to the PDGF- β receptor, nor has it any effect on the binding of antibody 163.3.1.1 to the PDGF- β receptor or on the ability of the antibody to block the binding of PDGF. It will be important to follow up these studies with a separate study looking at the effects of the

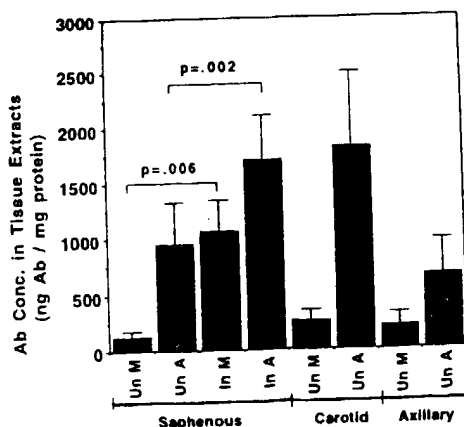


Figure 3. Quantitation of chimeric antibody 163.3.1.1 in artery wall extracts. ELISA was used to determine level of chimeric antibody in detergent-solubilized extracts of artery wall tissue. Arteries were microdissected into media (M) and adventitia (A) and extracted separately. Uninjured (Un) carotid and axillary arteries and uninjured and injured (In) saphenous arteries were evaluated.

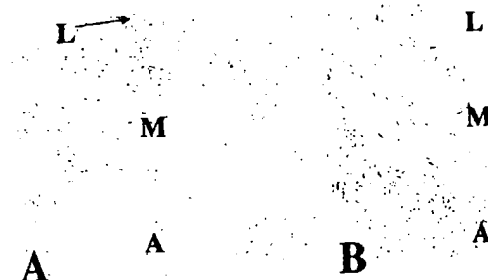


Figure 4. Cross sections of saphenous artery stained for chimeric antibody 163.3.1.1. Frozen sections obtained from animals treated for 4 days with chimeric antibody (study 2) were stained with a goat anti-human IgG₄ antibody to localize chimeric antibody in the artery wall. A, Noninjured saphenous artery; B, balloon-injured saphenous artery. L indicates lumen; M, media; A, adventitia. Bars=100 μ m.

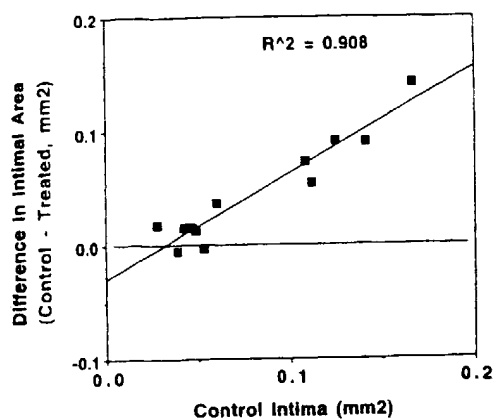


Figure 5. Correlation between control lesion size and effect of antibody treatment. Data were analyzed to monitor correlation between neointimal size in vehicle control-treated arteries and the effect of antibody treatment, the difference in intimal area between the vehicle control-treated and antibody-treated arteries (control intimal area minus Antibody-treated intimal area) for each individual animal. An r^2 value of 0.908 was obtained. Data demonstrate that those animals with largest lesions in control arteries had the greatest response to anti-PDGF- β receptor antibody treatment.

antibody alone in the absence of heparin. With the use of a synthetic vascular graft model in the baboon,²⁴ we have obtained preliminary data showing that the chimeric antibody alone is able to inhibit intimal hyperplasia (C.E. Hart and A.W. Clowes, unpublished observations, 1998).

In summary, the data obtained from this study indicate that PDGF plays a key role in regulating the extent of intimal hyperplasia at sites of acute vascular injury in the baboon. These findings have increased significance because they are one of the first studies to demonstrate an effective pharmacological strategy to inhibit intimal lesion development in a nonhuman primate model.

Acknowledgments

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Expression of platelet derived growth factor B chain and β receptor in human coronary arteries after percutaneous transluminal coronary angioplasty: an immunohistochemical study

Shinichi Tanizawa, Makiko Ueda, Chris M van der Loos, Allard C van der Wal, Anton E Becker

Abstract

Objective—To evaluate whether expression of platelet derived growth factor B (PDGF-B) protein is associated with expression of its receptor protein in human coronary arteries after angioplasty and to identify cells involved.

Background—PDGF is considered an important growth factor in the repair process of the vessel wall after angioplasty. In situ hybridisation has revealed expression of PDGF-A and -B chain messenger ribonucleic acid (mRNA) in human coronary arteries at sites of postangioplasty injury.

Methods—Target and non-target sites of eight coronary arteries were studied immunohistochemically for PDGF-B and PDGF- β receptor proteins in relation to macrophages, T lymphocytes, smooth muscle cells, and HLA-DR positive cells.

Results—The PDGF-B and PDGF- β receptor proteins were expressed in areas with distinct repair, containing α actin negative spindle cells, macrophages and, at later stages, α actin positive smooth muscle cells as well. When the neointima was composed mainly of α actin smooth muscle cells, PDGF-B expression was rare and PDGF- β receptor expression was negative.

Conclusions—There is expression of PDGF-B and PDGF- β receptor proteins at sites of postangioplasty repair in human coronary arteries. The associated cells are mainly macrophages and α actin negative spindle cells; the latter may be dedifferentiated smooth muscle cells. A link between PDGF expression and the postangioplasty time interval suggests a relation with cell differentiation as part of the maturation of the repair tissue. Mutual expression of both the growth factor and its receptor protein strongly suggests that in humans a PDGF mediated repair process occurs, with involvement of smooth muscle cells and macrophages.

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Keywords: restenosis; growth factors; smooth muscle cells; macrophages

The cellular response of the vessel wall after coronary angioplasty in humans is dominated

experimental studies it has been suggested that platelet derived growth factor (PDGF) is one of the biological determinants involved.⁴⁻⁷ The potential role of PDGF has also been highlighted by its presence in human atherosclerotic lesions.⁸⁻¹⁰ We have recently shown by in situ hybridisation techniques that PDGF-A and -B chain mRNA is expressed in human coronary arteries at sites of lesions after angioplasty (unpublished data). However, from the point of view of potential functional significance, it is essential to know whether the appropriate PDGF receptors are also expressed.

These data may improve our understanding of the postangioplasty wound healing processes in humans, since plaque morphology varies considerably. In some plaques the fibrous cap is dominated by smooth muscle cells, in others by macrophages, while the majority show intermediate varieties.¹¹ Furthermore, the type of laceration induced by the angioplasty procedure may also vary markedly.¹²⁻¹⁵ In view of the potential cellular heterogeneity present at the site of angioplasty injury, it is important to obtain information about the types of cell expressing PDGF. To do this, we have conducted a study using immunocytochemical techniques to identify cells expressing the PDGF-B protein and the PDGF- β receptor protein.

Methods

PATIENTS

The study is based on eight different dilated coronary arteries obtained from eight patients who had undergone an initial successful angioplasty, but who subsequently died and came to necropsy. All patients died as a result of ischaemic heart disease. The relevant clinical data are summarised in table 1.

The target site of the angioplasty procedure was identified by comparing the clinical angiograms with the heart specimens, taking the coronary ostia and bifurcation sites as points of reference. In two patients (case 7, left anterior descending coronary artery; case 8, left circumflex coronary artery) a follow up angiogram did not show evidence of restenosis. In the remaining six patients no follow up angiogram was done. None of the patients had immune deficiency.

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

All necropsies were performed within 3 h after

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Table 1 Relevant clinical data of eight patients following percutaneous transluminal coronary angioplasty (PTCA)

Case No	Age (years)	Sex	Reason for PTCA	Site of PTCA	Interval PTCA death	PTCA artery narrowing (% DR)		Cause of death
						pre	post	
1	78	F	AMI	RCA (1)	2 days	100	35	CHF†
2*	70	M	AMI	LAD (7)	6 days	100	33	Cardiac rupture†
3*	72	M	AMI	LAD (7)	14 days	100	42	CHF†
4*	67	M	AMI	RCA (2)	28 days	100	27	CHF†
5	58	M	AMI, OMI	LAD (6)	37 days	100	50	CHF†
6*	67	M	SAP, OMI	LAD (7)	44 days	90	10	CHF†, pneumonia
7*	58	M	AMI	LAD (6)	56 days	100	25	CHF
8	77	F	UAP, OMI	LCx (11)	6 months	99	40	CHF‡

AMI, acute myocardial infarction; CHF, congestive heart failure; DR, diameter reduction; LAD, left anterior descending coronary artery; LCx, left circumflex artery; OMI, old myocardial infarction; RCA, right coronary artery; SAP, stable angina pectoris; UAP, unstable angina pectoris.

Segment(s) of the coronary arteries that were dilated are shown in parentheses (according to the American Heart Association Committee Report. *Circulation* 1975; 51 [suppl]: 5-40).

*Material of these patients has been used for in situ hybridisation of PDGF-A and B chain mRNA.

†Relates to initial infarction.

‡Due to multivessel disease.

from the epicardial surface. The full length of the segments which had contained the balloon and which included the culprit lesion was then identified and the specimen was sliced serially at approximately 1 mm intervals. In cases 2, 3, 4, 6, and 7, one slice was fixed in methanol-Carnoy's fixative, a second slice was immersed in 4% paraformaldehyde, and a third slice was snap frozen. This sequence was repeated throughout the total length of the dilated arterial segment. In the remaining cases, a slice fixed with methanol-Carnoy's solution and a second snap frozen slice were repeated throughout the total length of the inflated segment. From the same arterial segment a distal smaller segment, remote from the area that had contained the balloon, was selected as control. The snap frozen samples were sectioned serially at 6 μ m thickness and fixed in acetone. Every first section was stained with haematoxylin and eosin; the other sections were used for immunocytochemical staining. Adjacent slices in 4% paraformaldehyde and in Carnoy's fixative were used for the evaluation of the site of angioplasty injury. The identification of angioplasty related injury was based on findings that a laceration continued through several slices and stayed geometrically at almost the same location in the artery. Moreover, the injury extended far beyond the area which contained the culprit lesion. On that basis we were certain that the sections selected for study contained angioplasty induced laceration.

The primary antibodies used are listed in table 2. For the identification of PDGF-B a mouse monoclonal antibody (PGF-007) was used (kindly provided by Mochida

Pharmaceutical Co, Inc, Japan), generated against a 25-amino-acid peptide located near the COOH terminus of human PDGF-B chain (residues 73 to 97 of mature B chain). Its specificity has been reported.¹⁶

For the identification of the PDGF- β receptor, a mouse monoclonal antibody (PDGFR-B2) was used (Oncogene Science). The antibody is raised against porcine PDGF receptor and recognises the extracellular portion of the human PDGF- β receptor (not the α receptor).¹⁶

Sections were incubated at 4°C overnight and then subjected to a three-step staining procedure, using the streptavidin-biotin complex method for colour detection. The other antibodies (see table 2) were incubated at room temperature for 1 h. Some sections of each case were double stained with HAM-56 (immunoglobulin M) and Leu 4 (immunoglobulin G1), HLA-DR (immunoglobulin G2a) and Leu 4 (immunoglobulin G1) or 1A4 (immunoglobulin G2a) and HAM-56 (immunoglobulin M), according to procedures previously reported.¹⁷

The specificity of the results obtained with PGF-007 and PDGFR-B2 was checked by omitting the primary antibodies and by using one irrelevant mouse immunoglobulin G antibody. Moreover, PGF-007 neutralisation with a relevant peptide (kindly provided by Mochida Pharmaceutical Co, Japan) was performed on frozen sections of human coronary arteries with advanced atherosclerosis, normal lung tissue (alveolar macrophages), and normal brain tissue (glial cells).

All single stained sections were counter-stained with haematoxylin.

Table 2 Source, specification, and working dilution of the antibodies

Designation	Specificity	Cell identified	Source reference	Working dilution
PGF-007	Amino acid residues 73 to 97 of mature PDGF-B chain	PDGF-B presenting cells	Ross <i>et al</i>	1:2000
PDGF-R-B2	Epitope present in the extracellular part of the PDGF- β receptor	PDGF- β receptor presenting cells	OS Rönstrand <i>et al</i> ¹⁶	1:200
HAM-56	—	Monocytes, macrophages some endothelial cells	Dako	1:50*
Leu 4	CD3	T cells	B & D	1:10*
1A4	α smooth muscle actin	Smooth muscle cells	Dako	1:50*
HLA-DR	Class II antigen	DR+ cells	B & D	1:25*

OS, Oncogene Science (Uniondale, New York, USA); Dako, Dako Laboratories (Glostrup, Denmark); B & D, Becton and Dickinson (Mountain View, California, USA).

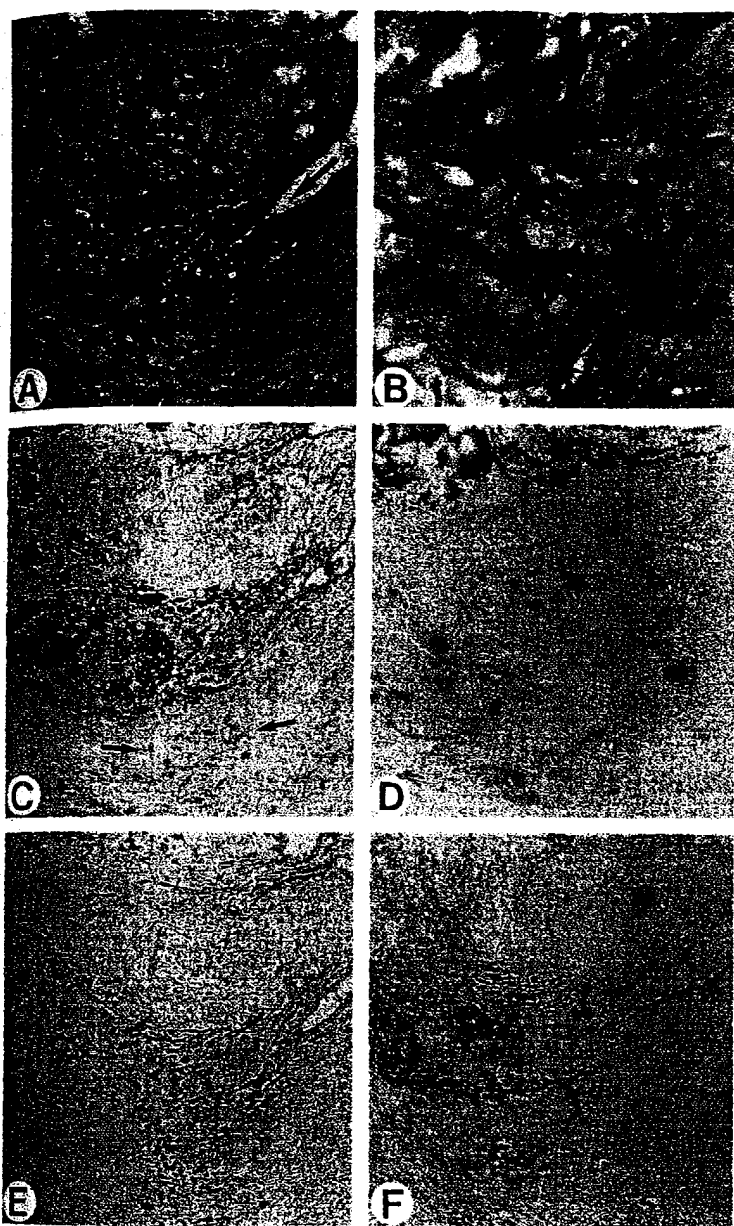


Figure 1 Micrographs of a postangioplasty injury site with a fissure into an atheroma, 14 d after PTCA. (A) Fissure (arrow) into an atheroma with intraplaque haemorrhage amid mononuclear cells (asterisk). The area delineated by the two small arrows is shown in higher magnification in (B). (B) Haematoxylin-eosin stain shows mononuclear cells and spindle cells immediately adjacent to the tear. (C) Immunodouble stain with an antimacrophage antibody (HAM-56: red) and an anti- α actin antibody (1A4: blue). At the site of plaque fissure, a large number of macrophages is seen. In the adjacent preexisting tissue, there are macrophages, smooth muscle cells, and (unstained) spindle cells. The area delineated by arrows is compatible with that shown in panels (A) and (B) and it is shown in higher magnification in (D). (D) Macrophages are stained; a few smooth muscle cells are faintly positive, but the majority of spindle cells do not stain with 1A4 or HAM-56. (E) PDGF-B stain. The macrophages which are seen along the plaque fissure stain positive. In the pre-existing adjacent tissue, macrophages, a proportion of the spindle cells (indicated by arrows in A), and some smooth muscle cells stain positive. (F) PDGF- β receptor stain. A proportion of macrophages at the area of plaque fissure shows positive staining. In the pre-existing adjacent tissue PDGF- β receptor positive cells correlate with macrophages and spindle cells (indicated by arrows in A); smooth muscle cells do not stain. Magnification (A, C, E and F), $\times 61.5$; (B and D), $\times 196$.

Results

Each of the eight dilated coronary arteries revealed pre-existing advanced atherosclerotic plaques at the target site. Wall laceration limited to the intima had occurred in six and had extended into the media in two.

TARGET SITE: INTIMAL INJURY

The artery at 2 d after angioplasty had a laceration at the site of a lipid-rich plaque. The

contained a thrombus extending into the ruptured plaque. HLA-DR positive monocytes/macrophages were present alongside the atheroma and within the thrombus. There was no staining for PDGF-B and PDGF- β receptor. The adjacent pre-existing tissue contained spindle cells, defined as cells not expressing α actin and negative for HAM-56, and a few cells identified as smooth muscle cells on the basis of a positive stain for α actin. A few spindle cells were HLA-DR positive. None of the cells stained for PDGF-B or for the PDGF- β receptor.

The artery at 6 d after angioplasty had a tear which also extended into an atheromatous area. The lesion contained many macrophages, which were mostly HLA-DR positive. A proportion of macrophages within the ruptured atheroma, and in particular those alongside the calcified border, stained positive for PDGF-B. However, none of the macrophages stained for the PDGF- β receptor. The adjacent pre-existing tissue contained macrophages and spindle cells, most of which were HLA-DR positive. A few smooth muscle cells were identified. Some of the macrophages were positive for PDGF-B and the PDGF- β receptor. The area which contained spindle cells stained positive for the PDGF- β receptor, but not for PDGF-B. The localisation was similar to that of the HLA-DR positive cells. None of the smooth muscle cells expressed PDGF-B or the receptor.

The artery at 14 d after angioplasty presented a ruptured plaque which contained a large number of macrophages amid plaque haemorrhage (fig 1). The macrophages stained positive for PDGF-B and some also stained positive for the PDGF- β receptor (fig 1). These cells were HLA-DR positive. The adjacent pre-existing tissue contained macrophages, spindle cells, and smooth muscle cells. Most macrophages were HLA-DR positive. Only an occasional spindle cell and smooth muscle cell showed HLA-DR positive staining. The areas that stained positive for PDGF-B coincided with those that stained positive for HLA-DR. PDGF- β receptor was expressed in the area containing macrophages and spindle cells, but no positivity for the PDGF- β receptor was found in an area containing mainly smooth muscle cells.

The arterial segment obtained at 28 d after angioplasty showed remnants of plaque thrombus, but there were now also spindle cells amid macrophages. The vast majority of these cells expressed HLA-DR. Most macrophages expressed PDGF-B, whereas only a proportion of the spindle cells expressed PDGF-B. Some of the macrophages and spindle cells were positive for the PDGF- β receptor. In the adjacent pre-existing tissues the cellular components were similar to those seen at 14 d. At this stage some of the smooth muscle cells also expressed PDGF- β receptor positivity.

The segment at 37 days after angioplasty showed a superficial injury of a lipid-rich lesion. Macrophages and spindle cells were

Figure 2 Micrographs of a postangioplasty injury site limited to the intima, 56 d after angioplasty. (A) Neointima (asterisk) at the site of injury (I, pre-existing intima). (B) Immunodouble stain with an antimacrophage antibody (HAM-56; red) and an anti-actin antibody (1A4; blue). The neointima is composed mainly of smooth muscle cells; only occasional macrophages are seen. (C) PDGF-B stain. In the neointima smooth muscle cells stain positive; a few macrophages in the neointima and in the pre-existing intima also show positivity. (D) PDGF- β receptor stain. The neointimal smooth muscle cells are negative; a few cells in the pre-existing intima show positive staining. Magnification $\times 148$.

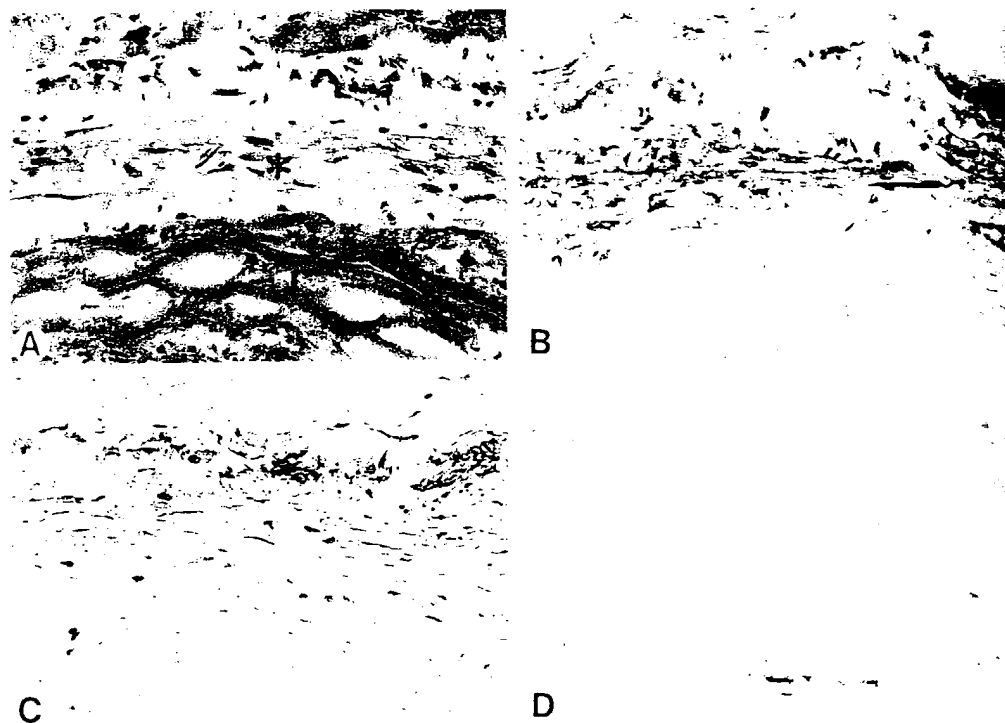


Figure 3 Micrographs showing a postangioplasty injury site with medial laceration, 44 d after angioplasty. (A) The laceration has extended from the intima (I) into the media (M). Fibrocellular tissue (asterisks) has filled the gap. The boxed area is enlarged in (B). (B) A higher magnification shows high cellularity in the neointima and the adjacent pre-existing intima. (C) Immunodouble stain with an antimacrophage antibody (HAM-56; red) and an anti-actin antibody (1A4; blue). The fibrocellular reaction is composed mainly of macrophages and smooth muscle cells. The boxed area is enlarged in (D). (D) Macrophages and unstained spindle cells with some neointimal smooth muscle cells are seen (compare to B). (E) PDGF-B stain. The same area shown boxed in (C). Smooth muscle cells and macrophages in the fibrocellular tissue stain positive. In the pre-existing intima, macrophages and some spindle cells stain positive. (F), PDGF- β receptor stain. The same boxed area shown in (C). Within the fibrocellular tissue, macrophages and some smooth muscle cells are positive. Within the pre-existing intima, macrophages and a proportion of spindle cells stain positive. There are more PDGF- β receptor positive spindle cells than PDGF-B positive cells (compare with E). Magnification (A, C, E and F) $\times 66$; (B and D) $\times 148$.

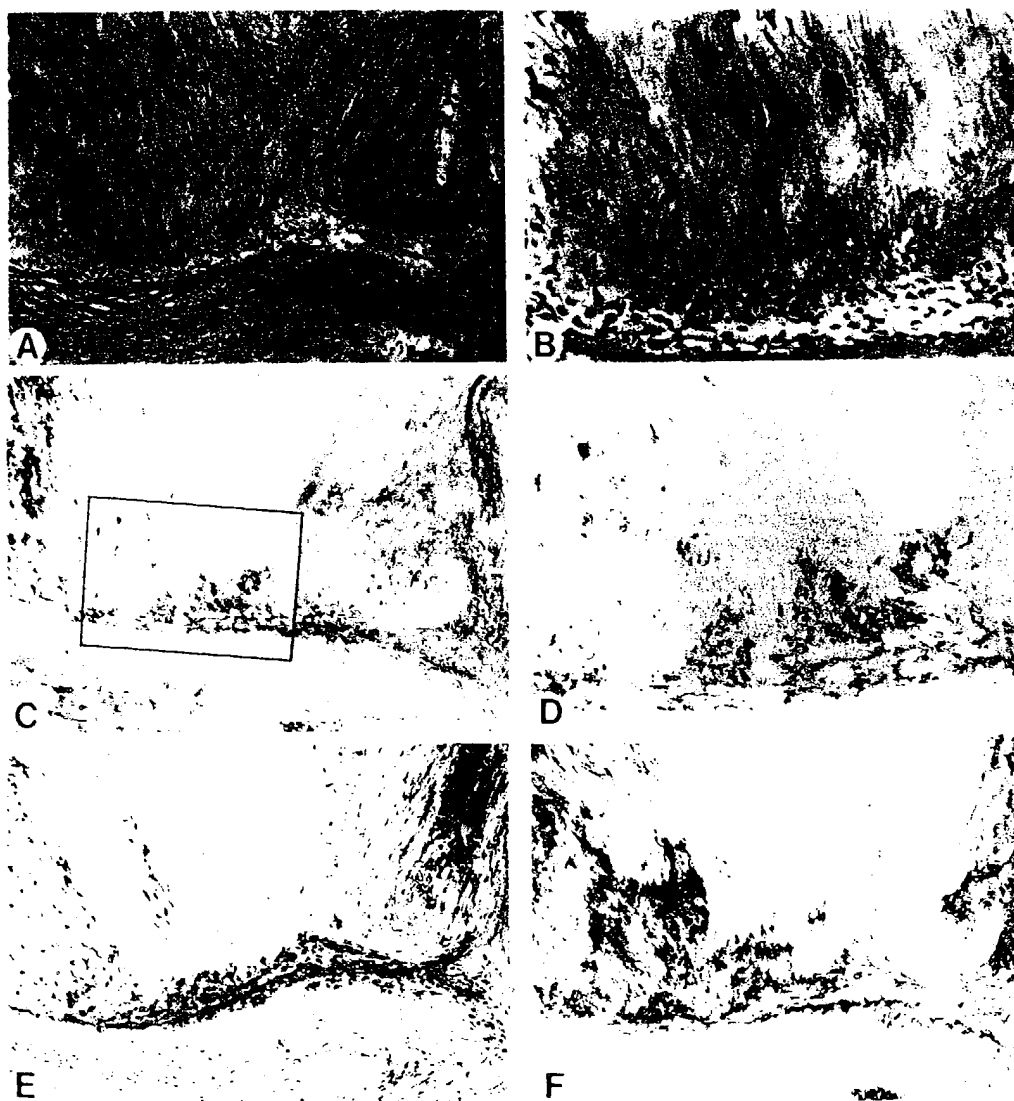
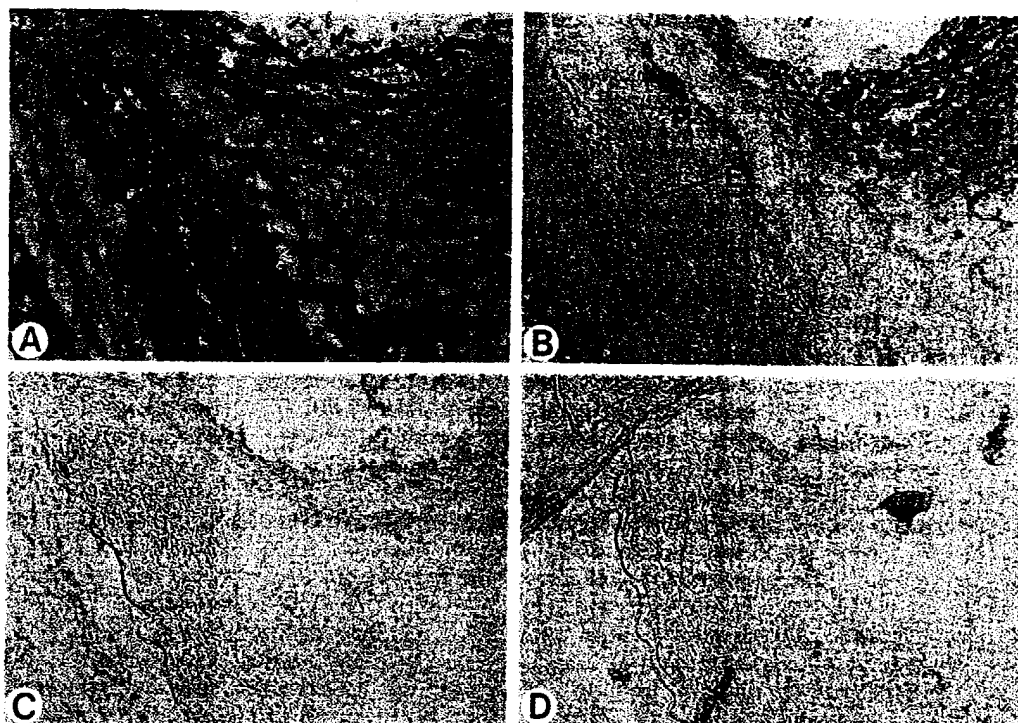


Figure 4 Micrographs showing a postangioplasty injury site with medial laceration, six months after PTCA. (A) Fibrocellular tissue (asterisk) at the site of medial laceration which separates the media (M) from the internal elastic lamina (EL). The fibrocellular tissue is present also at the luminal site (asterisk). (B) Immunodouble stain with an antimacrophage antibody (HAM-56; red) and an anti-smooth muscle α actin antibody (1A4; blue). The fibrocellular tissues (asterisks), sandwiched between the intima (I) and media (M) and at the luminal site, are composed mainly of smooth muscle cells. (C) PDGF-B stain. Some cells in the fibrocellular tissue show positive staining, but the majority are negative. (D) PDGF- β receptor stain. No positive staining in the fibrocellular tissue. Magnification $\times 75$.



was identified. Macrophages and spindle cells were HLA-DR positive. Positivity for PDGF-B and the PDGF- β receptor was found in the same area which contained macrophages and spindle cells and the occasional smooth muscle cell. Within the adjacent pre-existing tissues macrophages, spindle cells and a few smooth muscle cells were encountered. The majority of these cells expressed HLA-DR. Most macrophages and some spindle cells expressed both PDGF-B and the PDGF- β receptor. A few smooth muscle cells expressed PDGF-B or PDGF- β receptor or both.

The artery obtained at 56 days showed a superficial injury, covered by a distinct neointimal cap composed mainly of smooth muscle cells with only a few macrophages (fig 2). The latter were positive for HLA-DR, whereas only a few of the smooth muscle cells were HLA-DR positive. PDGF-B positivity was distinct and present in the same areas containing macrophages and smooth muscle cells (fig 2). PDGF- β receptor positivity was much less, but when present occurred in areas with macrophages. Smooth muscle cells were negative for PDGF- β receptor at this stage. In the adjacent pre-existing tissues, macrophages, spindle cells, and smooth muscle cells were encountered, with no HLA-DR staining among smooth muscle cells. Positivity for PDGF-B was encountered in the regions that coincided with the localisation of spindle cells, smooth muscle cells, and macrophages. The PDGF- β receptor was occasionally positive and related to areas containing macrophages and spindle cells. Smooth muscle cells were negative for the receptor.

T cells were distinct at the site of injury and always associated with macrophages. They were most pronounced at 28 and 37 d, but less so at 56 d.

INTIMAL/MEDIAL INJURY

The artery harvested at 44 d after angioplasty showed fibrocellular proliferation filling the laceration site. It was composed mainly of smooth muscle cells and macrophages (fig 3). Most macrophages and a proportion of smooth muscle cells were HLA-DR positive. Moreover, PDGF-B was strongly positive in the areas containing both macrophages and smooth muscle cells. Macrophages were strongly positive also for the PDGF- β receptor, and some of the smooth muscle cells also stained positive for the receptor. The adjacent pre-existing tissue contained macrophages which were distinctly positive for both PDGF-B and the PDGF- β receptor. Spindle cells, present in the pre-existing intima, were only occasionally positive for PDGF-B, but more cells stained positive for the PDGF- β receptor. The smooth muscle cells of the pre-existing media occasionally expressed PDGF-B, but there was no positivity for the PDGF- β receptor (fig 3).

The artery obtained at six months after angioplasty showed an extensive fibrocellular proliferation at the site of medial injury. The tissue was composed mainly of smooth muscle cells (fig 4). Only an occasional PDGF-B positive cell was seen; there was no staining reactivity for the PDGF- β receptor. Some smooth muscle cells were HLA-DR positive. In the adjacent preexistent tissues spindle cells were negative for PDGF-B, with only an occasional spindle cell showing positivity for the PDGF- β receptor. Smooth muscle cells, both in the intima and the media, were occasionally positive for PDGF-B, but none stained for the PDGF- β receptor.

DISTAL NON-TARGET SITE

These sites contained diffuse intimal thickening.

ing or mildly thickened fibrous intima, without appreciable lipid depositions and composed of smooth muscle cells amid collagen, with only a few scattered macrophages. Occasionally, some macrophages stained positive for PDGF-B; PDGF- β receptor staining was negative.

Discussion

In this study in humans we have shown that PDGF-B chain protein and its receptor protein are present at the site of injury in coronary arteries after angioplasty. Moreover, spindle and smooth muscle cells, as well as macrophages, appear to be associated with this PDGF mediated repair process.

A potential role of PDGF in the wound healing processes that occur after angioplasty injury has been suggested on the basis of observations in human atherosclerotic plaques⁸⁻¹⁰ and experimental studies.^{4,7} Both PDGF-A and -B, and their respective receptors have been incriminated.^{4,8} Recently the expression of PDGF-A and -B mRNAs in human coronary arteries after angioplasty has been documented using in situ hybridisation techniques (unpublished data). The present study has focused on the expression of PDGF-B and the PDGF- β receptor proteins only, because reliable monoclonal antibodies against PDGF-A and the PDGF- α receptor are not available to us.

PDGF-B PROTEIN

In this study, in the earliest stages after angioplasty available, all specimens had lacerations limited to the intimal plaque. In these instances monocytes/macrophages appear as the main source of PDGF-B protein. The appearance of HLA-DR positive monocytes/macrophages at two days after injury indicates active involvement, but at that stage the cells were still negative for PDGF-B. However, at six days after injury a proportion of macrophages located immediately adjacent to the tear expressed PDGF-B. Arterial segments harvested at subsequent later stages after angioplasty contained a higher ratio of PDGF-B positive versus PDGF-B negative macrophages, both at the injury site proper as well as in the adjacent pre-existing tissues. Late after angioplasty the number of macrophages was reduced and likewise the number of PDGF-B chain positive macrophages was minimal.

In addition to macrophages it appeared also that spindle cells (defined as cells not expressing α actin and negative with HAM-56) and smooth muscle cells (α actin positive) of the adjacent pre-existing tissue expressed PDGF-B protein; a phenomenon which in this study was identified first at 14 days after angioplasty. Once spindle cells appeared in the reactive tissue after angioplasty injury (at 28 days in this study) they also expressed PDGF-B protein. The same applied for smooth muscle cells, but their appearance in the neointima occurred at a later stage. Spindle cells were not identified in the neointima of the cases studied 44 days, 56 days, and six months after angioplasty, most

α actin containing smooth muscle cells.^{2,11} Smooth muscle cells within the neointima in this series were positive for PDGF-B for a considerable time, but at 6 months only an occasional positive cell was seen.

Previous immunohistochemical studies of human atherosclerotic plaques by Ross *et al*⁹ and Katsuda *et al*¹⁰ and of human wound healing tissue by Reuterdaahl *et al*,¹⁹ using the same monoclonal antibody PGF-007 as used in this study, showed that PDGF-B protein was expressed predominantly by macrophages. On the other hand, in situ hybridisation studies by Wilcox and associates⁸ led them to conclude that the predominant cell types expressing PDGF-B chain mRNA are mesenchymal-appearing intimal cells and endothelial cells, with little or no expression in macrophages. Our study differs from the studies reported by Wilcox *et al*,⁸ Ross *et al*,⁹ and Katsuda *et al*¹⁰ since these workers investigated the native atherosclerotic plaque, while we focused on postangioplasty repair processes. In view of these differences in study design, it is of interest that our observations strongly suggest that PDGF-B protein can be expressed by a variety of cells. The differences noted with previous studies could relate to differences in the type of tissue studied.

PDGF- β RECEPTOR PROTEIN

In the pre-existing tissues immediately adjacent to the site of injury, the expression of the PDGF- β receptor appeared first on spindle cells and macrophages. This phenomenon was seen as early as six days after injury. In contrast, smooth muscle cells of the adjacent pre-existing tissue did not express PDGF- β receptor at an early stage; positive cells were seen in the cases obtained at 28 and 37 days after angioplasty.

In the repair tissue at sites of injury limited to an atherosclerotic plaque, the PDGF- β receptor protein was expressed in the same area which contained macrophages. Once spindle cells were present in the repair tissue at the site of intimal injury, some of them also expressed the receptor. In the specimen with intimal injury at 37 days, a substantial number of spindle cells was positive for the PDGF- β receptor. In the specimen with medial injury at 44 days, the main cellular component of the repair tissue was identified as smooth muscle cell, and these cells expressed the PDGF- β receptor. At a later stage, however, smooth muscle cells within the repair tissue were negative for the PDGF- β receptor, both at the site of intimal injury and at sites of injury extending into the media.

Although previous studies in humans^{8,19,23} and in experimental animals^{24,26} have shown that different cells are capable of expressing the PDGF- β receptor, the present study is the first to demonstrate this phenomenon in human coronary arteries after angioplasty. The present findings suggest that the dominant cell type that expresses the PDGF- β receptor in postangioplasty repair tissue differs, depending on the type of injury

is limited to the intima or extends into the media—and possibly also on the stage of the repair process. In previous work we have shown that the cellular response after angioplasty is different when the injury is limited to the atherosclerotic plaque or extends into the media.^{15,27} We have shown also that at the site of medial injury dedifferentiation of smooth muscle cells occurs very soon after the injury, with reappearance of α actin positive cells at later stages of repair.¹⁸ The present study suggests that smooth muscle cells in the repair tissue express PDGF- β receptor only transiently during the evolution of the healing process after angioplasty. Rubin and coworkers²¹ investigated the expression of PDGF- β receptors in human blood vessels with abnormal vascular cell proliferation and concluded that a pronounced expression of PDGF- β receptors was seen on vascular smooth muscle cells in rejected kidneys, atherosclerotic carotid plaques, and chronic synovitis. Recently they have also reported that PDGF- β receptors are expressed by vascular smooth muscle cells in healing wounds of human skin.¹⁹ Our observations at least partially support these findings, but seem to indicate that α actin negative spindle cells play a more important role in expressing PDGF- β receptor. This observation endorses the findings of Wilcox *et al*⁸ who found PDGF receptor mRNA expression in mesenchymal-appearing intimal cells obtained from carotid arteries in humans. There is a real possibility of course that these spindle cells are basically derivatives of smooth muscle cells.¹⁸ Moreover, our observations in cases with neointimal proliferation further suggest that once spindle cells or smooth muscle cells differentiate into a matured phenotype they no longer express the PDGF- β receptor protein. This is of considerable interest since one could argue that at that stage PDGF is no longer actively involved as a growth factor in the wound healing process. In fact, one may hypothesize that the sooner the spindle cells or synthetic smooth muscle cells differentiate towards a more contractile phenotype, the sooner the proliferative response will come to a halt.

The mechanism involved in the induction of the receptor on the pre-existing spindle cells remains to be elucidated. However, the presence of activated macrophages at an early stage creates the possibility that these cells could act as intermediary by releasing cytokines like TGF- β , which is a potential inducer for the PDGF- β receptor.²⁸ In reactive cells, both at the injury site itself and in the immediately adjacent tissues, activated macrophages and T lymphocytes were always closely associated with spindle cells. This suggests active cellular interaction with a key role for PDGF. The results of a recent clinical trial, in which a PDGF antagonist was shown to reduce restenosis after PTCA, also support the concept that the postangioplasty repair tissue is PDGF mediated.²⁹

STUDY LIMITATIONS

In interpreting the findings it should be born

in mind that PDGF-B protein and the PDGF- β receptor protein are expressed in native atherosclerotic lesions.^{9,10,21} Hence the presence of the ligand and the receptor proteins at the site of injury could reflect a pre-existing condition rather than upregulation due to the insult. However, the appearance of the PDGF- β receptor on cells within the repair tissue strongly suggests "new" rather than "old" expression.

Similarly, it may be important that the injurious effect of the angioplasty procedure may differ from one lesion to another and that the procedure will affect pre-existing coronary atherosclerotic plaques with different morphologies. Hence, potential differences in the expression of PDGF-B protein and the PDGF- β receptor may occur. In other words, the expression of PDGF-B chain protein and the PDGF- β receptor protein may not necessarily be the same in all instances after angioplasty.

Caution is warranted, moreover, because the number of observations is limited to only eight time points and most of the angioplasty sites in this study had injury affecting an advanced atherosclerotic plaque. Thus further studies with more cases and different postangioplasty morphologies are needed to validate our observations.

During the course of this study Sinichi Tanizawa was a Research Fellow from the Osaka City University Medical School

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